ABSTRACT

DAVIS, ANNE SALLY. Improving Experimental Models for the Study of Human Influenza A Pathogenesis. (Under the direction of Jeffery K. Taubenberger and J. McHugh Law.)

Influenza A viruses cause substantial annual morbidity and mortality in the form of endemic or ‘seasonal’ epidemics and occasionally, pandemic viruses cause startling spikes in mortality such as in 1918. To date we lack the ability to predict the emergence of pandemic strains or even the evolution of seasonal strains. Developing better techniques for predicting emergence of new human-adapted viral strains and assessing these strains’ virulence is a global public health priority.

In this dissertation, I examined the binding and entry of human influenza A viruses in both archival tissue and a primary cell line in order to further our understanding of human influenza A pathogenicity, specifically the reconstructed 1918 pandemic influenza virus. First, I addressed, tissue autofluorescence in formalin-fixed, paraffin-embedded human respiratory tissue, an issue that hampers visualization of immunofluorescent markers in respiratory tract tissue. Second, I better characterized the \textit{in vitro} differentiated normal human bronchial epithelial (NHBE) cell model for analysis of human influenza infections by comparing it to formalin-fixed, paraffin-embedded human tissue samples derived from the same location in the human respiratory tract from which this primary cell line is harvested. Finally, I used the NHBE model to analyze the binding and entry of five 1918 pandemic influenza A viruses that differ only in their hemagglutinin receptor binding domain.

My research into diminishing autofluorescence in formalin-fixed, paraffin-embedded human respiratory tissues showed that Eriochrome black T, Sudan black B and sodium borohydride were differently efficacious in diminishing autofluorescence in these tissues and that the analysis techniques developed for this work are more broadly applicable to other tissue types. I also reported on the strengths and weaknesses of the NHBE model showcasing its similarities to its source tissue in terms of cell types and influenza receptors while cautioning against its use for multi-day infection studies due to epithelial integrity issues. Finally, my research with five 1918 H1N1 influenza receptor binding domain variants yielded
unexpected results wherein the “avianized” virus most efficiently entered both the ciliated and goblet type NHBE cells.
Improving Experimental Models for the Study of Human Influenza A Pathogenesis

by
Anne Sally Davis

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Comparative Biomedical Sciences

Raleigh, North Carolina

2014

APPROVED BY:

Jeffery K. Taubenberger
Committee Co-chair

J. McHugh Law
Committee Co-chair

Frederick Fuller

James H. Shelhamer
DEDICATION

First and foremost, I dedicate this achievement to my husband, Richard Mark Horner, who supports me in pursuing my dreams. They have involved a radical career change from international computer consulting to specialized veterinary medicine. I am forever grateful to the man who has stuck with me through my D.V.M., residency and Ph.D. It’s time to go surfing or perhaps fly your drones, My Love.

I also dedicate this work to Zoe and Robert Horner. Born together during my residency, they continue to inspire me daily with their individualistic fascination for our world. I re-learned that play is as important as work. Our best discoveries come through play.

My research mentor, Jeffery K. Taubenberger, has given me license to learn, fail, and most importantly learn from my failures on his budget, while still nagging me sufficiently to bring me to this finish line. He has renewed my interest in classical music; I play my clarinet again. Donna Bouley, since being assigned as my veterinary student mentor at Stanford Medical School for the summer of 2005, has remained my constant friend and mentor throughout, always reminding me there is a way through, so long as I set my mind to it.

Finally, I dedicate this work to my mom, dad, the rest of my family including our au pairs, and to all of my mentors and friends, particularly Kimberly MacDonald (in memoriam) and Anke Richter, who have supported me during this multi-year odyssey.
BIOGRAPHY

A. Sally Davis was born to a New Zealander and a Brit who met on a ship in the middle of the Pacific Ocean and settled initially in Canada. She immigrated to the United States with her family when she was 4 years old and having explored a good number of other countries as her home since, currently holds a US and NZ citizenship. Previously diagnosed by her peers with Serious Sesame Street Syndrome, she is very surprised to be completing this Doctor of Philosophy degree in as close to a single subject as she will likely ever get.

Sally is first a mother and wife, second a mentor and teacher, and third an experimental veterinary pathologist dedicated to improving human understanding of zoonotic infectious diseases. Her academic path has gamboled through computer science, archaeology, education, fine and culinary arts, biochemistry, biology, then into veterinary medicine. Her education at Dartmouth College has left her permanently interdisciplinary in her mindset. Her athletic path first rooted in competitive swimming, entered and maintains a love for volleyball and cycling, but has turned more recently to yoga including aerial yoga as well as triathlons, leaving her with an unexpected passion for running, particularly cross-country. She used to say that she would be found running only in the event that there was someone chasing her! This has taught her that ‘never’ is a dirty word. She is at home where there are people with whom she can do good work, share the experience, and build something new. Her path next takes her to Manhattan, Kansas where she plans to be the best Assistant Professor of Experimental Pathology on Earth. Best to shoot high, right?
I thank my Ph.D. committee for their support throughout the entire process of this Ph.D., including committee composition changes and any challenges introduced by distance. This work would have been impossible without the support of both the NIH's Comparative Biomedical Scientist Training Program run by Mark Simpson and Jeffery K. Taubenberger, in whose National Institute of Allergy and Infectious Disease Intramural lab, the Viral Pathogenesis and Evolution Section of the Laboratory of Infectious Diseases, I was based throughout the research portion of this Ph.D. Individual contributions to each research chapter are acknowledged within the respective chapters.
# TABLE OF CONTENTS

LIST OF TABLES .................................................................................................................... vi
LIST OF FIGURES ................................................................................................................ v

CHAPTER 1 Introduction ........................................................................................................ 1

CHAPTER 2 Characterizing and Diminishing Autofluorescence in Formalin-fixed
Paraffin-embedded Respiratory Tissue ................................................................................. 14
  Summary ....................................................................................................................... 15
  Introduction ................................................................................................................. 15
  Materials & Methods ................................................................................................. 16
  Results ........................................................................................................................ 20
  Discussion .................................................................................................................... 26
  Acknowledgements ..................................................................................................... 32
  References ................................................................................................................... 32
  Supplemental ............................................................................................................. 34

CHAPTER 3 Validation of Normal Human Bronchial Epithelial Cells as a Model for
Influenza A Infections in Human Distal Trachea ............................................................... 48
  Abstract .................................................................................................................... 49
  Introduction ................................................................................................................. 50
  Materials and Methods ............................................................................................. 52
  Results ........................................................................................................................ 69
  Discussion .................................................................................................................... 75
  Acknowledgements ..................................................................................................... 87
  Table .......................................................................................................................... 88
  Figures ........................................................................................................................ 89
  References ................................................................................................................... 117

CHAPTER 4 Binding and entry of 1918 Pandemic Influenza Receptor Binding
Domain Variants in Normal Human Bronchial Epithelial cells ........................................ 125
  Abstract .................................................................................................................... 126
  Introduction ................................................................................................................. 127
  Materials and Methods ............................................................................................. 131
  Results ........................................................................................................................ 137
  Discussion .................................................................................................................... 140
  Acknowledgements ..................................................................................................... 148
  Tables .......................................................................................................................... 149
  Figures ........................................................................................................................ 151
  References ................................................................................................................... 156

CHAPTER 5 Conclusion ...................................................................................................... 160
LIST OF TABLES

CHAPTER 2 Characterizing and Diminishing Autofluorescence in Formalin-fixed Paraffin-embedded Respiratory Tissue

Table 1. Treatment Abbreviations in their Order of Introduction ............. 17
Table 2. Comparison of Treatments for Diminishing Green Wavelength Autofluorescence in Human Respiratory Tissue ...................... 20
Table 3. ANOVA Connected Letters Reports for White Light Laser Excitation .................................................................................... 26
Table 4. ANOVA Connected Letters Reports for 458 nm ........................ 28
Table 5. ANOVA Connected Letters Reports for 405 nm ........................ 28

CHAPTER 3 Validation of Normal Human Bronchial Epithelial Cells as a Model for Influenza A Infections in Human Distal Trachea

Table. Microarray Presence/Absence Calls............................................. 88

CHAPTER 4 Binding and entry of 1918 Pandemic Influenza Receptor Binding Domain Variants in Normal Human Bronchial Epithelial cells

Table 1. 1918 Pandemic Influenza HA Receptor Binding Domain Variants Amino Acid Sequences....................................................... 149
Table 2. Hemagglutination assay results for AV, NY, and SC ............... 150
CHAPTER 2 Characterizing and Diminishing Autofluorescence in Formalin-fixed Paraffin-embedded Respiratory Tissue

Figure 1. Reduction of autofluorescence in formalin-fixed, paraffin-embedded human tracheal tissue by three treatments ................. 21
Figure 2. Λ² data collection and analysis process flow diagram .............. 23
Figure 3. Functional fit of Λ² data to 2D Gaussian surfaces .................. 24
Figure 4. Antigen retrieval and serum application do not increase autofluorescence in formalin-fixed, paraffin embedded human tracheal tissue in the 470-670 nm excitation range ..................... 25
Figure 5. Antigen retrieval and serum application do not increase autofluorescence in formalin-fixed, paraffin-embedded human tracheal tissue at 458 nm or 405 nm excitations ......................... 27
Figure 6. Eriochrome black T with antigen retrieval shifts formalin-fixed, paraffin-embedded human tracheal tissue autofluorescence to the right ................................................................. 29
Figure 7. Impact of antigen retrieval with Eriochrome black T is visible with 458 nm and 405 nm excitations .................................. 30
Figure 8. Sodium borohydride and Sudan black B are very effective quenchers of autofluorescence ............................................. 31
Figure S1. Output measurement at WLL pump power 100% vs. 60% ...... 38
Figure S2. Link to image array downloads ........................................... 39
Figure S3. Additional examples of multi-lambda data fit to 2D Gaussian form ................................................................. 40
Figure S4. Comparison of autofluorescence in FFPE human tracheal tissue treated with combinations of antigen retrieval and serum application ............................................................... 41
Figure S5. Antigen retrieval and serum application fail to increase autofluorescence (additional data) .............................................. 43
Figure S6. The effect of sodium borohydride is typically enhanced by AR and Se ................................................................. 44
Figure S7. Treatments quench autofluorescence in frozen human liver ..... 45
Figure S8. Shows the entire analysis process that was followed in this research ........................................................................... 46
CHAPTER 3 Validation of Normal Human Bronchial Epithelial Cells as a Model for Influenza A Infections in Human Distal Trachea

Figure 1  Normal Human Bronchial Epithelial cell types compared to human distal trachea and carina .................................................. 89
Figure 2. Electron microscopy of NHBEs and human carina ................. 90
Figure 3. Influenza receptor lectin distributions in NHBEs and distal trachea ................................................................................. 91
Figure 4. NY312-AF594 binding of NHBEs and distal trachea ............ 92
Figure 5. NY312 and CA04 NHBE infections ........................................ 93
Figure 6. Comparison of influenza antigen distribution by cell type
Pandemic 2009 influenza infected NHBEs and human trachea .... 94
Figure S1. Jacalin co-localized with Muc5Ac ...................................... 95
Figure S2. NHBE cell types, example 1 ............................................. 96
Figure S3. NHBE cell types, example 2 ............................................. 97
Figure S4. Distal trachea cell types .................................................... 98
Figure S5. Carina cell types ............................................................. 99
Figure S6. Average epithelial heights of distal trachea, carina and NHBE cells ................................................................. 100
Figure S7. Neuraminidase treatment removes sialic acids from NHBEs and distal trachea ............................................................. 101
Figure S8. *Sambucus nigra* on NHBEs ............................................. 102
Figure S9. *Sambucus nigra* on distal trachea sample 1 ...................... 103
Figure S10. *Sambucus nigra* on distal trachea sample 2 ................... 104
Figure S11. *Sambucus nigra* on carina matched with distal trachea sample 2 105
Figure S12. *Maackia amurensis* II on NHBEs .................................. 106
Figure S13. *Maackia amurensis* II on distal trachea sample 1 .......... 107
Figure S14. *Maackia amurensis* II distal trachea sample 2 .......... 108
Figure S15. *Maackia amurensis* II on carina matched with distal trachea sample 2 ................................................................. 109
Figure S16. *Maackia amurensis* I on NHBE cells and distal human trachea . 110
Figure S17. NY312-AF594 binding to NHBE cells ............................. 111
Figure S18. NY312-AF594 binding to distal trachea ........................... 112
Figure S19. NY312-AF594 and SNA compete for the same receptors .. 113
Figure S20. Influenza antigen distribution by cell type in CA04 Infected NHBEs ........................................................................... 114
Figure S21. Non-specific background signal with anti-influenza antibody labeling ................................................................. 115
Figure S22. Influenza antigen distribution by cell type in Pandemic 2009 influenza infected human trachea ................................... 116
CHAPTER 4 Binding and entry of 1918 Pandemic Influenza Receptor Binding Domain Variants in Normal Human Bronchial Epithelial cells

Figure 1. Comparison of influenza antigen distribution by cell type for 1918 receptor binding domain variants ................................................................. 151
Figure 2. Endosomal markers with influenza antigen ................................. 152
Figure 3. Replications kinetics for viruses AV, NY and SC ....................... 153
Figure 4. Real-time PCR results for all five viruses at a multiplicity of infection of 0.1 ................................................................. 154
Figure 5. Real-time PCR results for all five viruses at a multiplicity of infection of 0.01 ................................................................. 155
CHAPTER 1

Introduction
Influenza A viruses cause substantial annual morbidity and mortality in the form of endemic or ‘seasonal’ epidemics. Occasionally, pandemic viruses cause startling spikes in mortality such as in 1918 (Simonsen et al., 1998; Taubenberger and Morens, 2009; Wright et al., 2007). To date we lack the ability to predict the emergence of pandemic strains or even the evolution of seasonal strains (Taubenberger and Morens, 2006). Developing better techniques for predicting emergence of new human-adapted viral strains and assessing these strains’ virulence is a global public health priority.

While autopsy material can provide a rich resource for analysis of late infection, its single time point, an individual human’s death, limits the utility of this material. Accurate examination of stages of infections in humans via pathology is hampered by the inability to determine the infection start time or take tissue samples at selected time points. The mouse, ferret and to a lesser extent swine, guinea and a variety of non-human primates have been employed as experimental animal models for human influenza infections (Crisci et al., 2013; Louz et al., 2013; Thangavel and Bouvier, 2014). Each has its limitations.

The ferret is generally accepted as the best biological model of human influenza due to the close resemblance of clinical course of disease, viral transmissibility, and pathology to that in humans (Belser et al., 2011). Unfortunately, there is a dearth of ferret reagents thereby severely limiting the downstream analyses that may be done after an \textit{in vivo} infection. Additionally, some view the ferret to be overly permissive to influenza A infection, an \textit{in vivo} correlate to Madin Darby Canine Kidney epithelial cells, routinely used to amplify
influenza A stocks (personal communication Jeffery K. Taubenberger). Therefore, experimental results from this model should be interpreted with caution (Morens et al., 2012).

Consequently, other animals are commonly employed to examine questions unanswerable in ferret studies. Even though influenza transmission cannot be studied efficiently in mice, Balb/C and other common strains of mice are often used as experimental animal models for pathogenesis studies due to the extensive reagents available for work with them. Additionally, with mixed success, many different non-human primates have been tried because of their phylogenetic proximity to humans.

Prevailing hypotheses postulate that the hemagglutinin protein of avian-adapted influenza viruses binds host cells via $\alpha_{2,3}$ sialic acid (Sia)-linked glycans and that mammalian-adapted viruses bind $\alpha_{2,6}$ Sia-linked glycans, and that host switch requires a complete change in this Sia preference (Baigent and McCauley, 2003; Suzuki, 2005). These hypotheses are difficult to reconcile however with the frequent isolation from clinically ill patients of human influenza viruses that have mixed $\alpha_{2,3}/\alpha_{2,6}$ Sia binding specificities (Liu et al., 2010; Mak et al., 2010; Memoli et al., 2012; Rogers and Paulson, 1983) or zoonotic infection with avian influenza viruses.

A well-documented two amino acid change in the H1 hemagglutinin (HA) protein illustrates the contradictory nature of experimental findings. Stevens et al., 2004’s structural modeling
of the tertiary structures for the 1918 H1 HA receptor binding domain (RBD) revealed the α2,6 human form of the receptor to be more bulky than the α2,3 bird form of the receptor. A single change in the bird form’s RBD from glutamic acid (E) to aspartic acid (D) at position 187 is crucial for viral host switch from avian to mammalian whilst a second change at position 222 from glycine (G) to D switches sialic acid affinity from a mixed α2,3/α2,6 to completely α2,6 binding (Stevens et al., 2004). The current SIA hypothesis would predict that the 222D form would be better adapted to the mammalian host, transmit better, and may alter virulence. Indeed, Tumpey et al., 2007 found that the 222 D form transmitted more effectively in ferrets. However, infections of mice and ferrets with otherwise isogenic viruses with HA 222 G or D produced equivalent pathologic changes and viral replication (Qi et al., 2009). Furthermore, recent work on 1918 autopsy material, from which the RBD of HA protein was sequenced, also revealed equivalent pathology regardless of D or G at position 222 (Sheng et al., 2011).

Tumpey et al., 2007, proposed that the efficient transmission of the 222D virus is driven by the virus’ initial binding pattern. Affinity for SIA α2,6 provides a greater ability to bind the reportedly α2,6 dominated human upper respiratory tract. Therefore the transmission rate of viruses with a D at position 222 should be higher than those with a G at this position. Their ferret transmission data with the 1918 virus supports this hypothesis but the significance of the difference is difficult to assess given the small number of ferrets used per treatment group (Tumpey et al., 2007).
The comparable epidemiologic assessment for humans is the $R_0$, the reproductive number, which measures transmission efficiency (Wallinga and Lipsitch, 2007). Multiple studies based on clinical data have attempted to calculate $R_0$ for the 1918 influenza pandemic (Andreasen et al., 2008; Mills et al., 2004). Andreasen et al., 2008, estimated the $R_0$ for the spring-summer wave to be higher than that for the fall wave. Curiously, recent sequencing data based on 1918 autopsy material demonstrated that 222G was more common in the spring-summer wave case material (Sheng et al., 2011). Additionally, regardless of D or G at position 222, influenza viral antigen distribution in the respiratory tract as determined by immunohistochemistry (IHC) was qualitatively equivalent.

Interestingly, in three similar animal studies to those conducted by Tumpey et al., 2007, the equivalent D to G change in the 2009 H1N1 pandemic virus revealed no differences in transmission, viral lung titers, nasopharynx shedding, and pathology in ferrets (Belser et al.; Chutinimitkul et al.). However, two out of three similar studies in mice showed that the 222G virus caused greater weight loss and higher viral lung titers (Belser et al.; Chutinimitkul et al.; Zheng et al., 2010). Additionally, Zheng et al., 2010 showed that mice infected with 222G have a reduced lethal dose 50 (LD50), elevated pro-inflammatory cytokines in lung homogenates, and more severe histopathology. They attributed this increased virulence of the 222G virus to the virus’ mixed SIA binding capability providing a predilection for lower respiratory tract in humans. In preliminary studies, sequencing the HA RBD from viral RNA extracted from formalin fixed paraffin embedded (FFPE) tissue from several 2009 H1N1 pandemic autopsy cases revealed either D or G at the 222 position with no apparent pattern.
tied to severity of histopathology or presence of bacterial co-infection (Taubenberger lab, unpublished). In short, the predicted decreased transmissibility and increased lung disease severity of a 222G virus is not consistently supported by the available data.

Additionally, several *in vitro* experiments bring into question the exclusivity of SIA binding as a determinant of host cell infectivity. Stray et al., 2000 demonstrated that MDCK cells stripped of their SIA are still permissive to influenza infection and other *in vitro* studies identified other receptors that are critical for influenza infectivity regardless of the presence or absence of SIA glycans (Chu and Whittaker, 2004; Oshansky et al., 2011; Stray et al., 2000; Upham et al., 2010). In a study with viruses that were otherwise isogenic except for their HA, Qi et al., 2011 showed that otherwise isogenic viruses with pandemic virus HAs evade binding surfactant protein D, a type II pneumocyte product, regardless of their SIA binding preference, resulting in severe pathology in mice not observed with viruses bearing the HA of seasonal viruses (Qi et al., 2011). Sun and Whittaker, 2013 proposed that influenza may need a specific co-receptor for viral internalization or presentation of sialic acid on a specific protein (Sun and Whittaker, 2013). A reexamination of the role played by influenza SIA binding models in influenza biology is warranted.

My main hypothesis was that influenza A virus binding is driven by both variations in the receptor binding domain of the virus’ hemagglutinin protein as well as distribution of influenza A glycan receptors on target cells’ surfaces. Receptor-binding domain changes may fail, however, to produce a difference in late infection pathology and respiratory cell
type distribution of viral antigen for a sufficiently human-adapted virus. This suggests that otherwise isogenic human-adapted viruses that vary only in their preferences for α2,6 and α2,3 Sia will produce similar disease severity because their initial sialic acid binding pattern, while possibly predictive of cell types preferentially bound by the virus, does not adequately define the total susceptible cell population. Rather, additional mechanisms are likely at play.

Instead of employing an existing experimental animal model or developing a new one, I chose to tackle this hypothesis using human tissue and a human primary cell line that is differentiable into a pseudostratified epithelium in vitro resembling the tracheal epithelium. First, I improved our abilities to use archival human respiratory tissue for immunofluorescence work by characterizing autofluorescence that hampers its use and determining which methods best reduce this.

Second, I better characterized the selected primary cells, normal bronchial epithelial cells (NHBEs) for analysis of human influenza infections. Finally, I used this model to analyze a set of 1918 pandemic influenza A viruses that differed only in their HA receptor binding domain.

Researchers rely heavily on formalin-fixed paraffin-embedded tissue archives for research tissue samples and formalin is used as the main preservative for surgical and postmortem pathology tissue specimens. Tissue autofluorescence, a combination of natural tissue fluorescence and fixative and processing induced fluorescence, is present in most every fixed tissue and many fixed cells as well. I found no specific published guidance on diminishing
tissue autofluorescence in respiratory tissues. Therefore, I sought to characterize formalin-fixed paraffin-embedded human respiratory tissue autofluorescence and determine treatments that diminish it. I used both qualitative and quantitative techniques in this research.

NHBE cells are derived from the distal-most aspect of the human trachea, near the bronchial bifurcation also known as the carina. These cells are dedifferentiated post collection from cadavers. Researchers typically differentiate them into a pseudostratified epithelium prior to their use. They have been used for influenza infection studies. Differences between the source tissue from which they are derived and the redifferentiated epithelia may impact these infection studies. Therefore, I chose to compare these cells to their source tissue. This comparison included histologic and morphologic analysis by light and electron microscopy, a comparison of cell type distribution using fluorescence microscopy and microarray expression analysis, examination of influenza receptor distribution and an infection study comparison to autopsy tissue. In so doing, I sought to identify strengths and limitations in the NHBE model for influenza research. We hypothesized that the artificial environment in which the NHBEs are differentiated into epithelia might induce limitations. Knowledge of these limitations would improve experimental design capability with this model and suggest techniques for future improvements to the model.

Finally, I returned to the study of the D to G change at position 222 in HA receptor binding domain variants of 1918 (H1N1) pandemic influenza viruses. I compared the binding and entry of A/ SC/1/1918 (187D, 222D), A/NY/1/1918 (187D, 222G) and an “avianized” 1918
(187E, 222G) viruses. Additionally, within this experimental context, I examined two new 1918 receptor binding domain variants described in Sheng et al., 2011. The first, A/VA/1/1918, has an additional change from consensus Q (glutamine) to R (arginine) at position 189 and is predicted by molecular modeling prefer α2,6 sialic acid. The second, A/NY/3/1918, has predominantly N (asparagine) at position 222 and its binding preference is unknown. I hypothesized that these virus’ binding and entry profiles would align with their Sia preferences. An α2,6 preference would provide an advantage to a virus, seen as these viruses binding and entering the NHBE cells most readily.
References


CHAPTER 2

Characterizing and Diminishing Autofluorescence in Formalin-fixed Paraffin-embedded Human Respiratory Tissue
Characterizing and Diminishing Autofluorescence in Formalin-fixed Paraffin-embedded Human Respiratory Tissue

A. Sally Davis, Anke Richter, Steven Becker, Jenna E. Moyer, Aline Sandouk, Jeff Skinner, and Jeffery K. Taubenberger

Summary
Tissue autofluorescence frequently hampers visualization of immunofluorescent markers in formalin-fixed paraffin-embedded respiratory tissues. We assessed nine treatments reported to have efficacy in reducing autofluorescence in other tissue types. The three most efficacious were Eriochrome black T, Sudan black B, and sodium borohydride, as measured using white light laser confocal λ2 (multi-lambda) analysis. We also assessed the impact of steam antigen retrieval and serum application on human tracheal tissue autofluorescence. Functionally fitting this λ2 data to 2-dimensional Gaussian surfaces revealed that steam antigen retrieval and serum application contribute minimally to autofluorescence and that the three treatments are disparately efficacious. Together, these studies provide a set of guidelines for diminishing autofluorescence in formalin-fixed paraffin-embedded human respiratory tissue. Additionally, these characterization techniques are transferable to similar questions in other tissue types, as demonstrated on frozen human liver tissue and paraffin-embedded mouse lung tissue fixed in different fixatives. (J Histochem Cytochem 62:405–423, 2014)

Keywords
autofluorescence, formalin-fixation, paraffin-embedded tissue, immunohistochemistry, immunofluorescence, human, respiratory, confocal microscopy

Introduction
The successful visualization of fluorescent markers in formalin-fixed paraffin-embedded (FFPE) respiratory tissue sections is frequently hampered by tissue autofluorescence. Autofluorescence prevents the clean visualization of tissue-bound antibodies and lectins via immunofluorescence techniques, particularly when fluorochromes in the fluorescein isothiocyanate (FITC) range are used. The peak excitation of FITC is 500 nm and it emits at 75% relative intensity and greater from 506–532 nm (Life Technologies, Carlsbad, CA). Tissue autofluorescence has been attributed to many factors including endogenous tissue elements such as collagen, tissue processing techniques, particularly formalin fixation, and reagents such as serum that are applied to tissues during immunofluorescence protocols (Baschong et al. 2001; Billinton and Knight 2001; Collins 2006; Del Castillo et al. 1989). Key sources of autofluorescence in human tissue are likely lipofuscin, collagen, elastin, and red blood cells as well as formalin fixation (Banerjee et al. 1999;
Baschong et al. 2001; Billinton and Knight 2001; Collins 2006; Monici 2005; Viegas et al. 2007). Additionally, endogenous flavins, reduced nicotinamide-adenine dinucleotide and nicotinamide-adenine dinucleotide phosphate are common causes of autofluorescence in cell cultures, where the brighter extracellular proteins are absent (Billinton and Knight 2001; Monici 2005; Viegas et al. 2007). Additionally, serum blocking has been suspected to increase autofluorescence (personal communication with staff at the Bioimaging Section, RTB, NIAID, NIH) and antigen retrieval techniques may also play a role (personal communication with multiple researchers at the NIH). The little evidence that has been reported has largely been observational data.

Because the vast majority of tissues for human and animal pathology over the last century has been treated with FFPE, this study focused on reducing autofluorescence in typical FFPE sections. Unless prospective tissue collection for a study is possible, alternative fixatives such as paraformaldehyde, which has been reported to decrease autofluorescence in tissues (Clancy and Cauller 1998), are not generally an option. Our selection of treatments for diminishing autofluorescence in FFPE human respiratory tissue was guided by methods reported in the literature and recommended by other immunofluorescence practitioners for a diverse collection of tissue types (Baschong et al. 2001; Beisler et al. 1987; Callis 2006; Clancy and Cauller 1998; Collins 2006; Cowen et al. 1985; Kittelberger et al. 1989; Schenk and Churukian 1974; Schnell et al. 1999). To our knowledge, there has been no published study focused on which of these reagents work best on FFPE respiratory tissues.

This study focused on characterizing and altering the autofluorescence profile of FFPE human tracheal tissue; specifically, the epithelium, lamina propria, and submucosa, as these tissue layers are of highest interest to respiratory pathologists and virologists. We measured and modeled autofluorescence overall as well as endogenous, element-specific autofluorescence in FFPE human tracheal tissue, tested techniques for diminishing and shifting this autofluorescence, and evaluated changes to the tissue autofluorescence profile that the most promising of these techniques produced. We characterized the autofluorescence signature for FFPE tracheal tissue by measuring overall tissue autofluorescence using the $A^2$ mapping technique on a Leica SP5 white light laser (WLL) confocal microscope and performing mathematical modeling; specifically, 2D Gaussian surface fitting. Finally, we assessed processes reported to increase autofluorescence as well as the most promising treatments reported to decrease autofluorescence using these same techniques.

**Materials & Methods**

**Tissues**

Anonymous donor human respiratory tissues, both lung and trachea, were obtained from Capital Biosciences (Rockville, MD). Briefly, tissues were harvested from human cadavers at no more than several hours post-mortem and fixed with 10% neutral-buffered formalin, dehydrated, and embedded in paraffin. Approximately 5-µm-thick sections were cut and placed on positively charged slides. Similarly sectioned slides of HistoChoice (Sigma-Aldrich, St. Louis, MO) and of formalin-fixed paraffin-embedded (FFPE) mouse lung tissue were provided by the NIH Clinical Center Critical Care Medicine Department and NIAID Viral Pathogenesis and Evolution Section, respectively. Frozen cirrhotic human liver tissue sections were provided by the NIAID Hepatic Pathogenesis Section.

**Sample Preparation to Survey Techniques for Diminishing Autofluorescence**

Sections of both human trachea and human lung tissue were used in this initial comparison of treatments reported to diminish autofluorescence. The nine treatments were as follows: trypan blue, Eriochrome black T, Sudan black B, Chicago (Pontamine) blue, sodium borohydride, tris glycine, ammonium chloride, ammonia ethanol and UV transillumination. The first four, which are dyes, were hypothesized to either shift or mask the autofluorescence emission profile. The latter five are quenchers, hypothesized to decrease the intensity of the autofluorescence without shifting its emission. One physical and four chemical quenching techniques were tested. With the exception of the UV transillumination and ammonia ethanol treatments (detailed separately in this section), all slides were rehydrated and antigen retrieved as previously described (Martinez-Anton et al. 2013). The treatment of interest was then applied, $1\times$ TBST with 0.02% Tween-20 washes were performed to remove the reagent at the end of the treatment, and slide was mounted in SlowFade Gold antifade reagent with DAPI (Molecular Probes; Carlsbad, CA). For all treatments, a volume sufficient to cover the tissue throughout the incubation time (approximately 200 µl) was applied.

For many treatments, titrations of the reagent as well as various incubation periods were tested iteratively until all failed or a satisfactory result was obtained. All treatment incubations were completed at room temperature. Trypan blue (250 µg/ml) in pH 4.4-adjusted $1\times$ TBST was applied for 1 min (Collins 2006; Schenk and Churukian 1974). Eriochrome black T (EBT; 1.65%) was dissolved in DI water and applied for 5 min, as per personal communications with Robert Cunningham (formerly of the Armed Forces Institute of Pathology) (Kittelberger et al. 1989; Schenk and Churukian 1974). Sudan black B (SB) was prepared as 0.3% in 70% ethanol stained in the dark for 2 hr and then applied to tissues for 10 min (Baschong et al. 2001; Collins 2006; Schnell et al. 1999). To achieve the necessary staining translucency for bright field microscopy of the tissue, SB slides were rinsed briefly in 70% ethanol (EtOH)
followed by additional washing. Chicago blue was prepared at 0.5% in 1× TBS and then applied to tissues for 10 min (Collins 2006; Cowen et al. 1985). Sodium borohydride (NB) was prepared at 1 mg/ml in 1× TBS and kept on ice. It was applied to tissues for three consecutive 10 min incubations without any intermediary washes (Baschong et al. 2001; Beisker et al. 1987; Clancy and Cauller 1998; Collins 2006). Tris-glycine was prepared from 0.1 M glycine in TBS, adjusted to pH 7.4 using Tris, and applied to slides for 30 min (Callis 2006; Collins 2006). Ammonium chloride (50 mM) in 1× TBS was applied to tissues for 10 min (Callis 2006). After rehydration in 70% EtOH, a solution of 70% EtOH with 0.5% ammonia was applied for 1 hr, and then rehydration was resumed with the addition of a 50% EtOH step followed by distilled water. Slides were maintained in 1× TBSt between treatments. Slides were mounted in ProLong Gold. NB treatment was analyzed with the following human tracheal tissue slides were evaluated: no antigen retrieval (AR) without serum (No AR No Se), no AR with serum (No AR Se), AR with serum (AR Se), AR without serum (AR No Se). All treatment abbreviations used in the human tracheal tissue portion of this study are listed in Table 1. Regardless of treatment, all slides were rehydrated as per above. Slides that received AR and/or serum were processed as described followed by a 5 min 1× TBSt wash with several fluid exchanges in order to simulate washes that would occur during typical immunofluorescence protocols. Slides were maintained in 1× TBSt between treatments. Slides were mounted in ProLong Gold Antifade Reagent (Molecular Probes) as per vendor’s instructions. This change from SlowFade Gold with DAPI to Prolong Gold was deliberate, as the latter enables longer-term preservation of fluorescence. Additionally, samples were not stained with DAPI so that autofluorescence spectra could be collected with 405 nm excitation.

Sample Preparation to Measure Effect of a Select Number of Treatments

In order to examine the possibility that AR and serum application might interact with a downstream treatment, AR and serum application were continued in conjunction with the next two treatments, EBT and NB, which are targeted at diminishing autofluorescence. We applied EBT treatment to human tracheal sections in conjunction with AR and serum, which resulted in four combinations of treatments (see Table 1). The same procedure as detailed in the last section was followed for this slide production with the addition of the application of freshly prepared 1.65% EBT for 5 min after the serum application stage. Slides were then mounted in ProLong Gold. NB treatment was analyzed with the following

Table 1. Treatment Abbreviations in their Order of Introduction.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>No antigen retrieval and no serum</td>
<td>No AR No Se</td>
</tr>
<tr>
<td>No antigen retrieval and serum</td>
<td>No AR Se</td>
</tr>
<tr>
<td>Antigen retrieval and serum</td>
<td>AR Se</td>
</tr>
<tr>
<td>Antigen retrieval and no serum</td>
<td>AR No Se</td>
</tr>
<tr>
<td>Eriochrome black T, no antigen retrieval and no serum</td>
<td>EBT No AR No Se</td>
</tr>
<tr>
<td>Eriochrome black T, no antigen retrieval and serum</td>
<td>EBT No AR Se</td>
</tr>
<tr>
<td>Eriochrome black T, antigen retrieval and no serum</td>
<td>EBT AR No Se</td>
</tr>
<tr>
<td>Eriochrome black T, antigen retrieval and serum</td>
<td>EBT AR Se</td>
</tr>
<tr>
<td>Sodium borohydride, antigen retrieval and serum</td>
<td>NB AR Se</td>
</tr>
<tr>
<td>Sodium borohydride, no antigen retrieval and no serum</td>
<td>NB No AR No Se</td>
</tr>
<tr>
<td>Sudan black B, antigen retrieval and serum</td>
<td>SB AR Se</td>
</tr>
<tr>
<td>Sodium borohydride, Eriochrome black T, antigen retrieval and serum</td>
<td>NB EBT AR Se</td>
</tr>
<tr>
<td>Sodium borohydride, Sudan black B, antigen retrieval and serum</td>
<td>NB SB AR Se</td>
</tr>
</tbody>
</table>
combinations: NB AR with serum (NB AR Se) and NB No AR without serum (NB No AR No Se). We also looked at the effect of SB in conjunction with AR and serum (SB AR Se) to compare with EBT AR Se. Additionally, some combination treatments were explored, including NB with EBT (NB EBT AR Se) and NB with SB (NB SB AR Se) both with AR and serum. In the above preparations, serum was applied after NB whilst serum was applied before EBT and SB, as per previous recommendations (Baschong et al. 2001; Clancy and Cauller 1998; Kittelberger et al. 1989; Schenk and Churukian 1974; Schnell et al. 1999). Finally, in order to separate tissue-dependent results from treatment-dependent results, serial and/or step sections of AR Se, EBT AR Se, NB AR Se and SB AR Se were made.

Data Capture on the Leica SP5 White Light Laser Confocal System

Three fields, each always including regions of epithelium, lamina propria and submucosa, were analyzed for each of the 13 treatment combinations listed in Table 1. An additional experiment on triplicate sections with four of the treatments—AR Se, EBT AR Se, NB AR Se and SB AR Se—was also conducted in order to account for tissue section-dependent changes in the results. Data were collected with a 40×, 1.25 NA oil immersion objective at 600 Hz, bidirectional capture, 512×512 pixels, 387.50×387.50 μm field-of-view, pinhole 1 AU without any averaging or accumulation.

The WLL pump power was set at 60% directly on the laser unit. This compensation was done so that the measurement for the peak intensity of autofluorescence for all treatments fell within the range of the initial data and consequently was amenable to mathematical modeling. For more information on the WLL pump power, please see the Supplemental Methods.

A² WLL data was gathered in the xyλλ acquisition mode (Leica Microsystems 2010), with the following excitation/emission scan settings: excitation range 470–670 nm, λ-excitation step size 5 nm, detection range 480–780 nm, detection bandwidth 10 nm, and λ-detection step size 10 nm. During all sessions, the smart gain was 1020.8 and the smart offset -3.4%. Accompanying fluorescent images were collected for each data point. Post-data acquisition, excitation/emission scan contour plots and 3D views were produced in LAS AF Software (Leica Microsystems) for the A² data; this data was then exported for further processing in Origin Lab Pro version 9.0 (OLP) (OriginLab Corporation; Northhampton, MA) and Microsoft Excel (Microsoft Corp.; Redmond, WA).

Emission data at 405 nm and 458 nm were gathered using the UV diode and Argon lasers at 11% and 18%, respectively, and the xyλλ acquisition mode. For the 405 nm excitation, the following λ-scan range properties were employed: detection range, 415-775 nm; detection bandwidth, 10 nm; and detection step size, 10 nm. The 458 nm λ-scan settings were detection range, 470-770 nm; bandwidth, 10 nm; and step size, 10 nm. The 405 and 485 nm lambda data were also reviewed in LAS AF using the Quantify functionality and then exported for further analysis in Excel.

Images demonstrating autofluorescence levels in the green wavelengths and their change due to treatments were taken with a 40× oil objective, NA of 1.25 using the following settings on the WLL: pinhole, 1 AU; 387.50×387.50 μm field-of-view; 1024×1024 pixel format; line averaging, 4; HyD gain of 100%. An additional set of images demonstrating representative autofluorescence for AR Se, No AR Se, No AR No Se, and AR No Se were taken on a Leica SP8 confocal system with the following settings: pinhole, 1 AU, 387.50×387.50 μm field-of-view; 1024×1024 pixel format; line averaging, 4; HyD gain of 100%, with a 40× oil objective, NA of 1.25. For a given panel, the images were taken during a single session on serial sections of the same tracheal tissue. This was done in a separate session from the A² data capture because the images taken during those sessions are only 512×512 pixels. However, all A² session images and intensity data are provided in the form of image arrays produced in OLP from the LAS AF data.

Mathematical Modeling of A² Data

Data captured on WLL was imported into OLP. It was fit to a functional form using the Data Fitting command. Different functional form fits were compared using the Compare Models command, which calculates the Akaike’s Information Criterion (AIC) test. Functional forms that we considered were 2-dimensional (2D) Gauss, 2D LogNormal, 2D Voigt, 2D Lorentz, 2D Rational and 2D Gaussian. According to the AIC test, the Gaussian provided the best fit for the baseline data (AR Se, AR no Se, No AR Se, and No AR No Se) when compared against each of the other functional forms. This function form (2D Gaussian) was then maintained for the other treatments to permit cross treatment comparisons, even though certain treatments did have features that would have lent themselves to more complex functional forms with multiple peaks. Data reports for the model fits are provided as TIFF files exported from OLP. The 2D Gaussian function has seven parameters, but the four that were felt to be most biologically relevant were the location of the central peak of emission intensity (x₀, y₀) and the overall intensity of the emission, which was calculated from the floor of the functional form (z₀) and the peak intensity from this floor (A). Because fitting a 2D functional form is an estimation procedure that is a far more complex algorithm than with a typical 1D form, with slightly more or slightly less data it is possible to obtain a different model fit or for the fitting algorithm not to converge. To determine
the variations of model fit possible with the given data, two models at a minimum were fit for each data set collected. The models were created by progressively eliminating the low intensity levels, wherein the greatest noise was observed. For example, the first model would be fit using all of the available data; the second model would be fit using only data with an intensity greater than 1; and the third model would be fit only using data with an intensity greater than 2. If a model failed to converge, the next level of intensity would be eliminated and the model would be fit again. Because of this fitting procedure, the floor of the model \((x_c)\) would rise by necessity and hence, to obtain emission intensity levels, it is necessary to add this floor to the peak emission. As, for each treatment, three fields of data were captured, a total of at least six model fits were made per treatment. For each of these fits, the four model fit parameters were exported to Excel for further analysis. Additionally, graphics were created in OLP using both the raw data and the fitted models (as detailed in the next section).

**Examination of Fixed Mouse Lung Tissue Autofluorescence**

In another set of experiments, we compared otherwise untreated mouse lung tissue fixed with HistoChoice (Sigma-Aldrich; St Louis, MO) with mouse lung tissue fixed with 10% neutral-buffered formalin. We used the \(\Delta^2\) data collection techniques detailed above and then analyzed the resulting raw 3D data in OLP.

**Effect of Eriochrome Black T and Sudan Black B on Frozen Human Liver Tissue**

Experiments with cirrhotic human frozen liver tissue sections were used to examine the effect of EBT and SB on this tissue type. Three slides were prepared: untreated, EBT and SB. Frozen tissue slides were incubated in 100% ethanol for 10 min at -20°C. Without waiting for them to dry, they were rehydrated by washing in PBS with 0.01% Tween-20 (PBSt) twice for 5 min each. The slides were then blocked for 20 min with 10% normal goat serum diluted in PBS, washed in PBSt for 20 min, followed by two washes in PBS only. EBT or SB was applied as per the earlier described methods or the slide was left in PBS. Finally, slides were mounted in ProLong Gold. Similar to the mouse tissue analysis above, we used the \(\Delta^2\) data techniques to analyze the effect of the EBT and SB on this tissue type.

**Data Analysis**

Once the \(\Delta^2\) model data was imported into OLP the mean, standard deviation, and standard error emission intensity level were calculated for each treatment and plotted in a boxplot. In addition, scatter plots were created to permit comparison of the locations of the central peak of emission intensity \((x, y)\) for each model fit for each treatment. Smaller intensity levels equated to less autofluorescence with a value of zero representing an absence of autofluorescence detection at a given excitation/emission pairing. Relative emission intensity is unitless, but for presentation it is scaled and expressed using an 8-bit scale (0–255). Emission intensity is a function of both laser power and efficiency of excitation. The WLL output is not constant across all wavelengths. In order for the sample to be excited by constant power at each wavelength, the output of the laser is automatically adjusted. Therefore, the relative emission across the spectrum can be compared.

3D surface plots of raw WLL \(\Delta^2\) data as well as model output virtual matrices were created in OLP using Open GL 3D Surface Plot functionality. They enabled visual comparison of raw to modeled data for individual treatments, inter-field comparison for a single treatment and comparison of treatments in the form of 3D surfaces. Specifically, hybrid graphs composed of multiple color fill and matrix scatter surfaces were used to improve visual discrimination of this data. The choice of color fill vs. matrix scatter has no other significance with the exception of graphs that display raw data vs. model output, for which the former is a color fill surface and the latter a matrix scatter surface.

Data at 405 nm and 458 nm \(\lambda\) were directly imported into OLP for all analyses. Emission data for each excitation was graphed for three fields for each treatment slide and visually examined for differences in the shape of the emission intensity spectrum. The x and y-axes for these graphs were standardized within each laser excitation group. Because the 405 nm and 458 nm data were generated through the use of two different lasers, their intensity values were not comparable to each other. In addition, the maximum emission intensity was calculated for each field and the mean, standard deviation, and standard error for the maximum emission intensity was calculated for each treatment. Boxplots permit comparison of this data. Finally, the y-axes for the 405 nm, 458 nm and WLL intensity plots have different ranges on their y-axes. These are reflective of individual laser-dependent intensity ranges, which are also non-comparable between data sets.

In addition to the aforementioned summary statistics, we produced scatter plots of the locations of the central peak of emission intensity \((x, y)\) for each model fit of each treatment in Excel. We attempted to compare the locations of the central peaks using AIC comparisons, extra sums of squares F-tests, and multiple analysis of variance (MANOVA), but none of the results were satisfactory. Therefore, graphical assessment of the actual data was used instead to make comparisons among the different treatment options. For more details, see the Supplemental Section.
Intensities were compared among the 13 treatment groups (Table 1) using one-way ANOVA with Tukey post-hoc tests for multiple comparisons. This analysis was done in JMP 10.0.0 64-bit edition (SAS Institute; Cary, NC). As this is a comparison of all possible treatment pairs, we present the results in the form of a connected letters report (in Tables 3, 4, and 5), which shows the mean (average) maximum intensity for each treatment as well as the groupings into which each of the treatments were categorized. Treatments not sharing a letter are significantly different from each other; treatments sharing a letter are not significantly different.

Confocal images presented in figure panels were post-processed in Imaris 7.6.3 (Bitplane; Zurich, Switzerland). However, session images, which were exported in TIFF format from the Leica software and uploaded directly into OLP for image array assembly, were not post-processed in Imaris.

### Results

#### Three Treatments Significantly Diminish Green Wavelength Autofluorescence

Nine treatments, previously reported to reduce autofluorescence in a diverse range of cells and tissue types, were semi-quantitatively graded for their ability to reduce autofluorescence in FFPE human respiratory tissue. These treatments were also assessed for their level of interference with visualization on other channels such as DAPI (75% intensity emission range 431–492 nm) and rhodamine red (75% intensity emission range 582–602 nm) (Life Technologies), as well as ease of preparation and reproducibility of the treatment (Table 2). A 0–3 scale was used to grade the reduction in autofluorescence, from no appreciable reduction (0) to marked reduction (3); to describe degree of interference with other channels, including bright field visualization, DAPI, and rhodamine red visualization, and MP excitation from 0 (no interference perceived) to 3 (marked interference perceived), with the descriptor characterizing the interference; and variability in ease of preparation and reproducibility of the technique over multiple protocol runs from 0 (complex to prepare and hard to reproduce a consistent result) to 3 (easy to prepare and highly reproducible).

EBT diminished tissue autofluorescence in the green wavelengths (Fig. 1A, 1B). However, it shifted (i.e., moved the highest intensity components of) this autofluorescence from the green to red wavelengths. NB was the most effective quencher, diminishing yet not shifting the autofluorescence (Fig. 1C). SB, although more labor intensive in its preparation and its application, also showed promise (Fig. 1D). It appeared to mask the autofluorescence rather than shifting it. UV treatment showed minimal change, likely because the range of UV exposures did not adequately overlap with that of the tissue autofluorescence. The other quenchers and dyes either failed entirely, despite titrations of concentration and extensions of incubation time, or had only minimal effect, markedly less than the three aforementioned treatments.

EBT, NB and SB, the most promising treatments for diminishing autofluorescence on human respiratory tissue, were thus selected for further study. We also added steam antigen retrieval and serum application to our study set because both are frequently used in immunofluorescence protocols and concerns had been raised regarding each of
them contributing to an increase in autofluorescence. The full set of treatments for this section of the study and their acronyms is listed in Table 1. Finally, because the differences between autofluorescence level change on lung and tracheal tissue were minimal, the tissues to be tested were narrowed to human tracheal tissue for the remainder of the study.

**Mathematical Modeling of $\lambda^2$ Data is a Valid Technique for Comparing Treatment Effects**

In order to compare treatment-induced changes in human tracheal tissue autofluorescence, we captured autofluorescence intensity images using Leica $\lambda^2$-technology on the

---

**Figure 1.** Reduction of autofluorescence in formalin-fixed, paraffin-embedded (FFPE) human tracheal tissue by three treatments. Comparison of green wavelength autofluorescence (AF) in tracheal FFPE tissue slides that received antigen retrieval and serum application +/- AF diminishing treatment: (A) untreated control, (B) Eriochrome black T, (C) sodium borohydride and (D) Sudan black B. Images were taken on a Leica SPS WLL confocal system and are presented as maximum projections of 11.33-µm-sized z-stacks; excitation was with an Argon laser (488 nm) at 4% and detection range was 504–553 nm. White asterisks denote the tracheal lumens. Arrows denote red blood cells within vessels and extravasated red blood cells. Bar: 20 µm.
Leica SP5 WLL confocal system (Leica Microsystems 2010). For each excitation, a full series of emission measurements was made for the entire field. A 2D intensity map and corresponding image was constructed for each excitation/emission combination so that all the intensity data could be reviewed in a single 3D surface graph. With this approach, for each pixel, the full photonic range was captured, not just the peak intensity. Given our data collection settings (as described in the Materials & Methods), for each field analyzed, we generated 769 images of autofluorescence with all possible excitation/emission combinations. We provide these images paired with their intensity values in image arrays, one per field measured (Supplemental Fig. S2A–2NN). In order to compare these data in a more quantitative fashion than direct visual inspection of individual images or via the 3D surface generated for each field, we fitted the data to 2D Gaussian surfaces and focused on model outputs with biological significance. All model fit reports are provided as supplemental data (Supplemental file: “Model Fits.docx”). The model fit outputs of biological significance were central peak of emission intensity (x, y) and the overall intensity of the emission, which was calculated from the floor of the functional form (z) and the peak intensity from this floor (A). The overall process of data collection and analysis for a single field on one treatment slide is summarized in Figure 2 (and in Supplemental Fig. S8).

The raw A² data for both AR Se fields and EBT AR Se fields fit the 2D Gaussian model (Fig. 3A, 3B; Supplemental Fig. S3A–S3D), and the model fit similarly across raw data sets within a treatment (Fig. 3C, 3D). This demonstrated that the model fit the most disparate treatments reasonably well (i.e., no treatment and EBT). We, therefore, compared treatment results at the model level instead of at the raw data level.

In order to determine if inter-slide variation would necessitate the use of multiple tissue sections per treatment, we compared multiple slides (non-serial sections) from the same tissue block for a sub-set of treatments: AR Se, EBT AR Se, NB AR Se and SB AR Se. We found that the intra-slide field variation was, at times, greater than the inter-slide field variation. Therefore, we considered a single tissue section heterogeneous enough to represent autofluorescence ranges expected for a given treatment on multiple sections cut from the same tissue block.

**Steam Antigen Retrieval and Serum Application Have Minimal Effect on Tissue Autofluorescence**

As we suspected from our visual review of images, modeling and analysis revealed that AR and Se application did not impact the autofluorescence signature on otherwise untreated tissues across the channels analyzed: 470–670 nm WLL, 458 nm and 405 nm excitations. Representative green wavelength tissue autofluorescence images for these four treatments are shown in Supplemental Figure S4A–S4D. 3D surface plots of representative model fits of the A² data for each of the four treatments—AR Se, No AR No Se, AR No Se and No AR Se—showed minimal variation in model fit (Fig. 4A). A scatterplot of x vs. y revealed that the central peaks of emission intensity were tightly grouped and overlapping for all four treatments; two model fits for all three fields from each treatment are shown (Fig. 4B). A box plot of intensity levels (sums of z, and A) also showed overlap for these four treatments (Fig. 4C) consistent with the ANOVA results showing no statistical difference (Table 3). The increased intensity in the serum application surfaces seen in the representative model comparison (Fig. 4A) averaged out to no difference across all data sets (Fig. 4C; Supplementary Fig. S5A–S5D) and was therefore not significant (Table 3). Additionally, there was little variance in the central peak of the autofluorescence emission and intensity levels at 458 nm and 405 nm excitations (Fig. 5A–5D and Tables 4, 5).

**Eriochrome Black T Shifts Tissue Autofluorescence with Antigen Retrieval Only**

We found the role of AR to be critical when using EBT but that serum application made no appreciable difference. 3D surface plots of representative model fits of the A² data for each of the four EBT treatments—EBT AR Se, EBT No AR No Se, EBT AR No Se, and EBT No AR Se—showed that only EBT AR Se and EBT AR No Se applications resulted in a striking right shift (increase in wavelength) of the central peak of intensity (Fig. 6A). This finding was also seen in a scatterplot of the central peaks of emission intensity where EBT treatment with AR groups separated from EBT treatments without AR (Fig. 6B). The WLL intensity box plot shows that the average intensity levels of the EBT AR-treated slides were twice that of the EBT non-AR slides, and that the EBT non-AR treatments were equivalent to the non-EBT AR treatments discussed earlier (Fig. 4C and Table 3). A comparison of EBT AR Se to AR Se revealed a right shift in autofluorescence wherein tissue autofluorescence was markedly decreased in the green wavelengths emission range but increased in the red wavelengths emission range (Fig. 6C) and the scatterplot of AR treatments with and without EBT showed distinct groupings of the EBT AR slides and non-EBT AR slides (Fig. 6D). The 458 nm and 405 nm excitations intensity boxplot data for the four EBT treatments showed no statistical differences in mean intensity levels between these treatments (Fig. 5C, 5D and Tables 4, 5). However, review of the full emission profiles for both of these revealed a partial shift in autofluorescence in all slides treated with EBT and AR (Fig. 7A, 7B).
Figure 2. A^2 data collection and analysis process flow diagram. The flow chart depicts the entire analysis process that was followed in this research. The process starts in the upper left hand corner with the creation of the treatment slides. Following the arrows leads to two different scanning processes that were used to analyze the slides. For each of the scanning processes, different assessments and evaluation techniques were employed. One scanning process created 2-dimensional data which was analyzed with line graphs and box plots of the maximum intensities. The other scanning process created 3-dimensional (3D) data. The scanning software itself produces several images and output arrays (the two right-most arrows out of “LAS AF A^2 scans”) and the data were also analyzed by mathematical software that allowed us to fit surfaces to the data to permit the comparison of the different treatments. This allowed us to create 3D surface plots as well as assess and compare the location of the central peak and the relative intensity observed at this peak for each of the treatments. ANOVA analysis was used to compare the maximum intensities between treatments to determine if there were any significant differences. This is summarized in the connected letters reports.
but no shift in those treated with EBT with no AR (Fig. 7C, 7D). Additionally, this right shift was an obvious shift from baseline for the EBT AR treatments; e.g., AR No Se vs. EBT No AR No Se vs. EBT AR No Se at both excitations (Fig. 7E, 7F).

**Sodium Borohydride Dampens Autofluorescence Universally**

As demonstrated in representative 3D surface plots, NB markedly dampened autofluorescence across the spectra, regardless of the presence of AR and Se (Fig. 8A). Moreover, AR and Se applications appeared to enhance this effect (Fig. 4C and Supplemental Fig. 6); however, this finding was not significant (Table 3). Although, the central peak of intensity did not move, grouping with AR Se (Fig. 8B), the change in mean intensity following NB treatment was significant, along with SB AR Se (see below) (Fig. 4C). These treatments grouped statistically separately from AR Se and there was no significant difference in the two NB treatments’ groupings (Table 3). The line graphs of 458 nm and 405 nm excitation emissions also revealed decreases in autofluorescence (Fig. 8C, 8D) and the intensity averages for both NB treatments grouped together (Tables 4, 5).

**Sudan Black B Quenches Autofluorescence, and Combined Treatments Yield Mixed Results**

SB AR Se had a similar result as NB, with a greater magnitude of autofluorescence intensity change (Fig. 8E, 8B, and 4C). However, there was no significant difference between SB AR Se and the two NB treatments (Table 3). Similar changes to those above for NB were also seen with 458 nm and 405 nm excitations (Fig. 5C, 5D), where SB clearly lowered autofluorescence intensity more than that seen for any of the single treatments; yet it still grouped statistically with the two single NB treatments (Tables 4, 5). Finally, the excitation of the SB-treated

---

**Figure 3.** Functional fit of $\Lambda^2$ data to 2D Gaussian surfaces. Three-dimensional (3D) surface plots of raw $\Lambda^2$ data fit to the 2-dimensional (2D) Gaussian model: (A) Antigen retrieval (AR) and serum (Se) and (B) Eriochrome black T (EBT) AR Se. Data points: 1230 data points (pts) is the full raw data set; 810 pts is the next step in data point removal. The model fits generated from multiple data collections within a treatment align well: (C) AR Se models representing all three fields and (D) likewise for EBT AR Se. Filled and dot matrix surfaces are used solely for better visualization and have no additional meaning here or in later use in figures.
slide with an MP laser resulted in boiling of the SB during excitation, an effect not observed with the EBT- or the NB-treated slides.

Combining SB and NB was no better than either treatment alone while combining EBT and NB appeared to result in further dampening of autofluorescence compared with these treatments alone, albeit, this latter finding was not statistically significant, as NB EBT AR Se grouped consistently with NB AR Se (Tables 3-5).

**Treatments Tried on Frozen Liver Show Similar Results, and Mouse and Human Lung Have Similar Autofluorescence Profiles**

Difficulty visualizing a FITC-conjugated antibody in frozen cirrhotic human liver tissue led to an opportunity to compare the autofluorescence diminishing capability of EBT and SB in a different human tissue type. As in human lung tissue, EBT application shifted the autofluorescence signal

---

**Figure 4.** Antigen retrieval (AR) and serum (Se) application do not increase autofluorescence in formalin-fixed, paraffin-embedded human tracheal tissue in the 470–670 nm excitation range. A comparison of models and model outputs for AR Se, No AR No Se, AR No Se and No AR Se shows little difference in the four treatments. (A) Representative model fits. (B) A scatter plot of model variables x, y reveals that, for all model fits for all four baseline treatments, the central peak location of emission intensity is similar. (C) A box plot of all means of maximum intensity (sum A and z0) for all treatments collected by white light laser excitation 470–670 nm reveals a complete overlap for the four baseline treatments. Bars are SE (red) and SD (black). Intensity is a unitless, laser-dependent value.
to the right whereas SB lowered the intensity of the autofluorescence signal without shifting the central peak of emission (Supplemental Fig. S7). Additionally, we found mouse lung tissue as did mouse lung fixed in the similar autofluorescence signature to that seen in equivalent human tracheal tissue that is fixed in neutral-buffered formalin and then embedded in paraffin has an autofluorescence profile that fits a 2D Gaussian surface with a central peak of intensity with an excitation in the 470 nm and emission in the 530 nm (No AR No Se tissue). This aligns the peak autofluorescence well with the emission of FITC, thereby explaining the difficulty with FITC immunofluorescence on this tissue type, a commonly reported finding (Baschong et al. 2001; Beisker et al. 1987; Callis 2006; Clancy and Cauller 1998; Cowen et al. 1985; Kittelberger et al. 1989; Neumann and Gabel 2002; Schenk and Churukian 1974).

In this study, neither steam AR with a pH 6 citrate buffer nor application of 5% donkey serum increased autofluorescence significantly. We did not explore steam AR with EDTA or alternative pH citrate buffers. Additionally, we did not explore microwave-, enzymatic- or pressure cooker-based AR. We suspect that the latter would be the same, as we have found anecdotal evidence that both pressure cooker- and vegetable steamer-based AR are quite comparable in their effects on tissue samples. Also, we did not explore other blocking reagents; therefore, there is the possibility that other blocking reagents might increase autofluorescence. Moreover, if excess quantities of these blocking reagents are not removed in subsequent washes, their residues might also increase autofluorescence. In order to more successfully visualize fluorescent markers of interest, we used a pH 6 citrate buffer and serum application—both common steps in immunofluorescence protocols—did not increase autofluorescence. A number of treatments to decrease tissue autofluorescence were evaluated, and we found that EBT, SB, and NB were the most effective at diminishing tissue autofluorescence. In order to more quantitatively study the efficacy of these treatments, we performed and shown to be an effective technique for the analysis of treatment efficacy.

The treatments we did not carry forward into further analysis were not all ineffective on respiratory tissues, and those that were ineffective, might be effective when used on other tissue types with different endogenously autofluorescent elements. For example, lipofuscin, an autofluorescent red blood cell breakdown product found in hepatocytes, is not present in respiratory tissue (Schnell et al. 1999). Additionally, our use of light irradiation to diminish autofluorescence was restricted to a narrow UVB band (280–320 nm) quite distant from the peak excitations we measured in the 400-nm range. The broader band irradiation of tissue presented by Neumann and Gabel (2002), although attractive and likely quite efficacious, was not feasible due to facility limitations.

As captured using $\chi^2$ analysis on the Leica SP5 WLL confocal system, human tracheal tissue that is fixed in neutral-buffered formalin and then embedded in paraffin has an autofluorescence profile that fits a 2D Gaussian surface with a central peak of intensity with an excitation in the 470 nm and emission in the 530 nm (No AR No Se tissue). This aligns the peak autofluorescence well with the emission of FITC, thereby explaining the difficulty with FITC immunofluorescence on this tissue type, a commonly reported finding (Baschong et al. 2001; Beisker et al. 1987; Callis 2006; Clancy and Cauller 1998; Cowen et al. 1985; Kittelberger et al. 1989; Neumann and Gabel 2002; Schenk and Churukian 1974).

In this study, neither steam AR with a pH 6 citrate buffer nor application of 5% donkey serum increased autofluorescence significantly. We did not explore steam AR with EDTA or alternative pH citrate buffers. Additionally, we did not explore microwave-, enzymatic- or pressure cooker-based AR. We suspect that the latter would be the same, as we have found anecdotal evidence that both pressure cooker- and vegetable steamer-based AR are quite comparable in their effects on tissue samples. Also, we did not explore other blocking reagents; therefore, there is the possibility that other blocking reagents might increase autofluorescence. Moreover, if excess quantities of these blocking reagents are not removed in subsequent washes, their residues might also increase autofluorescence.

EBT is easy to apply and consistent in shifting autofluorescence from the green to red wavelength emission range when applied to steam AR-treated tissues. We are uncertain of the basis for this observation, but EBT as a complex-sensitive indicator can complex with Ca$^{2+}$ and other divalent metals thereby changing from blue to red (Dubenskaya and Levitskaya 1999). Additionally, EBT has a polar group and therefore would be expected to have its effects in the water-soluble portion of the sample. The shift from the green to red wavelength resembles an excitation energy transfer from the autofluorescent species to a new chromophore—the bound EBT perhaps. If so, then EBT or modified EBT absorbs the autofluorescence emission, thereby reducing its intensity, then emits at the higher wavelength.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR Se</td>
<td>B</td>
</tr>
<tr>
<td>No AR Se</td>
<td>B C</td>
</tr>
<tr>
<td>No AR No Se</td>
<td>B C</td>
</tr>
<tr>
<td>AR No Se</td>
<td>B C D</td>
</tr>
<tr>
<td>EBT AR No Se</td>
<td>A</td>
</tr>
<tr>
<td>EBT AR Se</td>
<td>A</td>
</tr>
<tr>
<td>EBT No AR Se</td>
<td>B C D E</td>
</tr>
<tr>
<td>EBT No AR No Se</td>
<td>B C D E</td>
</tr>
<tr>
<td>NB AR Se</td>
<td>D E F</td>
</tr>
<tr>
<td>NB No AR No Se</td>
<td>C D E F</td>
</tr>
<tr>
<td>SB AR Se</td>
<td>E F</td>
</tr>
<tr>
<td>NB SB AR Se</td>
<td>F</td>
</tr>
<tr>
<td>NB EBT AR Se</td>
<td>B C D</td>
</tr>
<tr>
<td>AR Se</td>
<td>B</td>
</tr>
<tr>
<td>EBT AR Se</td>
<td>A</td>
</tr>
<tr>
<td>NB AR Se</td>
<td>D E F</td>
</tr>
<tr>
<td>SB AR Se</td>
<td>E F</td>
</tr>
<tr>
<td>NB SB AR Se</td>
<td>F</td>
</tr>
<tr>
<td>NB EBT AR Se</td>
<td>B C D</td>
</tr>
</tbody>
</table>

These ANOVA results support the data shown in Fig. 4C. Treatments not sharing a letter (grouping) are significantly different from each other (p<0.05). Treatments sharing a letter are not. Abbreviations: AR, antigen retrieval; Se, serum; EBT, Eriochrome black T; NB, Sodium borohydride; SB, Sudan black B.
lower wavelength than the blue form, is more likely to be the absorbing species (Duhenskaya and Levitskaya 1999). AR may expose epitopes for EBT binding, thereby producing a new fluorescent species. Alternatively, EBT may form this species by reacting with a residual component from the AR step. At times we noticed that the green to red wavelength shift was incomplete, which presented as a small second peak in the WLL data (Fig. 3B). Additionally, dual peaks were present consistently on the 458 nm and 405 nm emission graphs (Fig. 7A and 7B). Therefore, increased EBT concentrations may diminish green wavelength autofluorescence even further.

Based on the model outputs, the post-treatment intensity for EBT AR with or without Se was significantly stronger than the original peak autofluorescence in the green wavelengths. Although this may be true, it is more likely that the intensity in the red wavelengths is the same as or less than that seen prior to treatment in the green wavelengths due to an innate characteristic of the WLL system. Power output of the WLL, particularly at the 60% level used to enable good mathematical model fits across all treatments in this project, varied by wavelength (Supplemental Fig. S1). Therefore, a comparison of treatments that diminish autofluorescence without shifting the central peak of this autofluorescence is straightforward. However, comparing treatments that shift autofluorescence to those that do not or those that shift it differently is difficult. We did not undertake the additional data collection needed to examine the entire profile of WLL power variation. Additionally, it is unclear how these data might be used to normalize intensity. So, although we are confident that the EBT shifted the autofluorescence peak to the right, we are uncertain whether the peak is more, lower wavelength than the blue form, is more likely to be the absorbing species (Duhenskaya and Levitskaya 1999).

Figure 5. Antigen retrieval (AR) and serum (Se) application do not increase autofluorescence in formalin-fixed, paraffin-embedded human tracheal tissue at 458 nm or 405 nm excitations. Emission line graphs for the four baseline treatments (AR Se, No AR No Se, No AR Se, and AR No Se) at 458 nm excitation (A) and 405 nm excitation (B); box plots of the means of maximum intensity (sum A and z0) for all treatments at 458 nm excitation (C); and 405 nm excitation (D) reveal little variance at 458 nm and 405 nm excitations for the four baseline treatments. Bars on box plots are SE (red) and SD (black). Intensity is a unitless, laser-dependent value.
equivalently, or less intense, as the WLL power applied to the tissue increases as one moves from the high 400s nm excitation to the mid-500s nm (Supplemental Fig. S1). Consequently, this implies that the significant ANOVA findings, where EBT AR Se and EBT AR No Se are grouped differently from all other treatments, must be interpreted cautiously.

We can, however, examine single windows of this intensity increase issue by examining the 458 nm and 405 nm excitations. At both 458 and 405 nm excitations, the EBT treatments did not group separately from the four AR, Se combinations, indicating that this intensity in the red wavelengths for EBT AR Se and EBT AR No Se is likely equivalent in intensity to the green wavelength autofluorescence seen for the four AR, Se combinations and the EBTs that did not receive AR. Thus, we recommend EBT as a treatment for autofluorescence in combination with AR so long as one does not plan to use a marker that emits in the red wavelengths.

NB diminished autofluorescence without changing the location of the central peak of its emission. Its primary mechanism of action for diminishing autofluorescence is via a reduction in the introduction of aldehydes and amine-aldehydes from formalin fixation to the respective alcohols (Baschong et al. 2001; Clancy and Cauller 1998). We recommend NB as a treatment for diminishing autofluorescence. However, its use is a bit more involved than EBT, requiring a chemical hood and attended incubations. If left on for too long or in too high concentration, the tissue may detach from the slides.

The effect of SB on tissue autofluorescence mimicked that of NB. As with EBT, we are not certain of its mechanism of action. Unlike EBT, SB is lipid soluble. It absorbs at 580 nm (Thakur et al. 1989) so may absorb a small amount of tissue autofluorescence. Additionally, its quenching action may be attributable to absorption of autofluorescence, direct chemical interactions with autofluorescent species, or other causes. Preparation and application of SB is, again, more involved than that of EBT. In particular, the post-incubation removal of the reagent is a fine dance between leaving residual black particulate matter adhered to the slide and removing all dye and, consequently, its autofluorescence-diminishing effect. Additionally, we observed boiling of residual SB when the tissue was excited by a multi-photon laser.

In summary, we show that bright-field microscopy image clarity varied between the three treatments: NB, EBT and SB. We did not discern a difference between AR Se- and NB AR Se-treated tissues under the bright-field

### Table 4. ANOVA Connected Letters Report for 458 nm Excitation.

<table>
<thead>
<tr>
<th>Treatment Grouping</th>
<th>Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR Se</td>
<td>A B C</td>
</tr>
<tr>
<td>No AR Se</td>
<td>A B</td>
</tr>
<tr>
<td>No AR No Se</td>
<td>A</td>
</tr>
<tr>
<td>AR No Se</td>
<td>A B C D</td>
</tr>
<tr>
<td>EBT AR No Se</td>
<td>D E F</td>
</tr>
<tr>
<td>EBT AR Se</td>
<td>B C D E</td>
</tr>
<tr>
<td>EBT No AR Se</td>
<td>E F G</td>
</tr>
<tr>
<td>EBT No AR No Se</td>
<td>C D E</td>
</tr>
<tr>
<td>No Se</td>
<td></td>
</tr>
<tr>
<td>NB AR Se</td>
<td>E F G H</td>
</tr>
<tr>
<td>NB No AR</td>
<td>E F G H</td>
</tr>
<tr>
<td>SB AR Se</td>
<td>F G H</td>
</tr>
<tr>
<td>NB SB AR Se</td>
<td>H</td>
</tr>
<tr>
<td>NB EBT AR Se</td>
<td>G H</td>
</tr>
<tr>
<td>AR Se</td>
<td>A B C</td>
</tr>
<tr>
<td>EBT AR Se</td>
<td>B C D E</td>
</tr>
<tr>
<td>NB AR Se</td>
<td>E F G H</td>
</tr>
<tr>
<td>SB AR Se</td>
<td>F G H</td>
</tr>
<tr>
<td>NB SB AR Se</td>
<td>H</td>
</tr>
<tr>
<td>NB EBT AR Se</td>
<td>G H</td>
</tr>
<tr>
<td>SB AR Se</td>
<td></td>
</tr>
<tr>
<td>NB SB AR Se</td>
<td></td>
</tr>
<tr>
<td>NB EBT AR Se</td>
<td></td>
</tr>
</tbody>
</table>

These ANOVA results support the data shown in Fig. 5C. Treatments not sharing a letter (grouping) are significantly different from each other ($p<0.05$); treatments sharing a letter are not. Abbreviations: AR, antigen retrieval; Se, serum; EBT, Eriochrome black T; NB, sodium borohydride; SB, Sudan black B.

### Table 5. ANOVA Connected Letters Report for 405 nm Excitation.

<table>
<thead>
<tr>
<th>Treatment Grouping</th>
<th>Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR Se</td>
<td>A B</td>
</tr>
<tr>
<td>No AR Se</td>
<td>A B</td>
</tr>
<tr>
<td>No AR No Se</td>
<td>A</td>
</tr>
<tr>
<td>AR No Se</td>
<td>A B C D</td>
</tr>
<tr>
<td>EBT AR No Se</td>
<td>C D E F</td>
</tr>
<tr>
<td>EBT AR Se</td>
<td>B C D E</td>
</tr>
<tr>
<td>EBT No AR Se</td>
<td>B C D E</td>
</tr>
<tr>
<td>EBT No AR No Se</td>
<td>C D</td>
</tr>
<tr>
<td>NB AR Se</td>
<td>B C D E</td>
</tr>
<tr>
<td>NB No AR No Se</td>
<td>B C D E</td>
</tr>
<tr>
<td>SB AR Se</td>
<td>E F</td>
</tr>
<tr>
<td>NB SB AR Se</td>
<td>F</td>
</tr>
<tr>
<td>NB EBT AR Se</td>
<td>D E</td>
</tr>
<tr>
<td>AR Se</td>
<td>A B</td>
</tr>
<tr>
<td>EBT AR Se</td>
<td>B C D E</td>
</tr>
<tr>
<td>NB AR Se</td>
<td>B C D E</td>
</tr>
<tr>
<td>SB AR Se</td>
<td>E F</td>
</tr>
<tr>
<td>NB SB AR Se</td>
<td>F</td>
</tr>
<tr>
<td>NB EBT AR Se</td>
<td>D E</td>
</tr>
</tbody>
</table>

These ANOVA results support the data shown in Fig. 5D. Treatments not sharing a letter (grouping) are significantly different from each other ($p<0.05$); treatments sharing a letter are not. Abbreviations: AR, antigen retrieval; Se, serum; EBT, Eriochrome black T; NB, sodium borohydride; SB, Sudan black B.
**Figure 6.** Eriochrome black T (EBT) with antigen retrieval (AR) shifts formalin-fixed, paraffin-embedded human tracheal tissue autofluorescence to the right. AR is required to achieve an effect with EBT. (A) 3D surface plots of representative models of the four treatments (EBT AR with Serum (Se), EBT AR without serum (No Se), EBT No AR No Se, EBT No AR Se). (B) Scatter plot of central peaks of emission for all EBT model fits. (C) 3D surface plots of model for EBT AR Se vs. AR Se. (D) Central peak of emission scatter plot for AR treatments with and without EBT.

Microscope. EBT AR Se-treated tissue elements were lightly outlined in black whereas SB AR Se-treated tissues were not only outlined in darker black stain but also had residue that frequently blocked the visibility of tissue elements even after thorough wash steps. We found no significant additional diminishment of autofluorescence when using SB or EBT in combination with NB. Consequently, neither approach was considered to be efficacious in this study. However, on another tissue type, or even on respiratory tissue with additional optimization of the concentrations or incubation steps, a combination treatment might yield an improvement over the treatment with the single reagent.

These findings can be extrapolated to FFPE human respiratory tissues in general based on our observations during the 9-treatment comparison wherein treatment effects on lung mirrored those on trachea. We also saw that the untreated tissue autofluorescence signature of mouse lung mimicked that seen in human respiratory tissues, irrespective of whether the tissue was fixed in neutral-buffered formalin or HistoChoice. Likely, the autofluorescence treatment responses in the wider mouse respiratory tract would be similar to those that we saw in human trachea. The application of the mathematical modeling technique presented here would enable validation of this hypothesis. Finally, elements of this study provide a starting point for improving our understanding of autofluorescence in other tissue types regardless of their preparation. The autofluorescence profile of frozen human liver was similar to that of human trachea; the application of EBT and SB also yielded similar results.

The technology and methodologies employed in this study have broader applicability. We regularly use *A* analysis to ascertain marker(s) visibility above background.
Figure 7. Impact of antigen retrieval (AR) with Eriochrome black T (EBT) is visible with 458 nm and 405 nm excitations. (A-B) Emission profiles for EBT and AR at 458 nm and 405 nm excitations, respectively. There is a partial shift in autofluorescence observed for EBT and AR-treated slides at 458 nm and 405 nm excitations. (C-D) Emission profiles for EBT and No AR at 458 nm and 405 nm excitation respectively. There is no shift observed with EBT treatment with AR is absent. (E-F) The EBT AR No Se emission profiles group separately from the EBT No AR Se and No AR Se profiles at the 458 nm and 405 nm excitation readings. This is seen as a partial right shift of the central peak of emission, visible as a double-peaked structure more prominently at 458 nm excitation than at 405 nm excitation.
Figure 8. Sodium borohydride (NB) and Sudan black B (SB) are very effective quenchers of autofluorescence. Regardless of presence of antigen retrieval (AR) in the protocol, NB markedly quenched autofluorescence. (A) Comparison of models of NB with or without AR and serum (Se) to AR Se. (B) Central peaks of emission scatter plot showing there is no shift in the center of the tissue autofluorescence. This quenching of autofluorescence is also present at 458 nm and 405 nm excitations, respectively (C-D). A comparison of AR Se-based treatments alone (E) shows representative diminishment of autofluorescence by each treatment (AR Se, EBT AR Se, NB AR Se and SB AR Se).
autofluorescence with and without additional tissue treatments to diminish auto fluorescence. These techniques might also be extended to analyses of shifts in a tissue’s auto fluorescence signature due to disease processes wherein an autofluorescent tissue component’s organization and distribution is modified. Such work would first require better separation of the individual endogenous contributors to the tissue auto fluorescence and the ability to identify them separately within the model. Such are the goals of the numerous studies focused on tissue fluorescence spectroscopy as well as techniques such as those reviewed in Cicchi et al. (2013) for collagen and elastin (Cicchi et al. 2013; Monici 2005).

In this study, we assessed several treatments for diminishing auto fluorescence. Throughout, we used novel quantitative techniques that, to the authors’ knowledge, have not been previously reported in the literature, including the development of a mathematical model for demonstrating the changes in auto fluorescence. These techniques should prove broadly useful for investigating similar questions in different species, tissue types and even tissue preparations, as shown by our experimentation with FFPE mouse lung and frozen human liver tissues. In conclusion, we found EBT, NB and SB to be differently efficacious in diminishing auto fluorescence in FFPE human respiratory tissue.

Acknowledgments

We thank Sandra Horton and her staff at the North Carolina State University College of Veterinary Medicine Histopathology Laboratory for their preparation of tissue sections for immunohistochemistry and auto fluorescence treatments; Robert Cunningham, formerly at the Armed Forces Institute of Pathology, for his auto fluorescence treatment suggestions and support; Marta Melis at the Hepatic Pathogenesis Section, Laboratory of Infectious Diseases, NIAID for frozen liver tissue samples treated with Eriochrome black T and Sudan black B; Joe Kovacs and Geetha Kuty at the Critical Care Medicine Department, Clinical Center, NIH for providing HistoChoice-fixed paraffin-embedded mouse lung tissue slides; Alice Haddy, Professor Biophysical Chemistry, University of North Carolina Greensboro, for her insights into the mechanisms of action for EBT and SB; Katherine Davis for figure panel compilation assistance; and Owen Schwartz and Lily Koo of the NIAID Research Technology Branch Bioimaging Section for their technical assistance with the diverse types of microscopy employed in this study. ASD and JKT are further thankful for the support of the NIH Comparative Molecular Pathology Research Training Program. This work was completed in partial fulfillment of A. Sally Davis’ dissertation work towards a PhD in Comparative Biomedical Sciences at North Carolina State University College of Veterinary Medicine.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was supported by the intramural research funds of the NIH and the NIAID.

References


Supplemental Methods

Adjustment of the WLL pump power

The WLL pump power is typically set at 100% on the Leica SP5 system used in this study. However, the laser is still functional at lower power levels, and for example, it is set at 75% on the newer Leica SP8 systems. The 60% setting, used in this study that provided consistent mathematical modeling was found empirically. Since all measurements for all treated slides were then retaken at the comparable 60% power WLL setting in a single session Measurements across the output spectrum were then taken for WLL power readings at 100% and 60% (Fig. S1) in order to describe the impact that the WLL power adjustment made. These measurements were graphed using Microsoft Excel version 14.3.2. Over time the relative power level across the output spectrum of the WLL changes, with a marked decrease in the 470-490nm. Consequently, data sets gathered temporally far apart are likely not comparable. If multiple sessions of data capture are required, the following method should be applied before each session to ensure that equivalent WLL power levels are employed. A constant power curve calculation can be run before the beginning of each
session. This will compensate for any wavelength dependent decrease in power as the laser ages. This curve can then be saved in the confocal software and applied during data collection.

Mathematical Modeling and Statistics

There are multiple sources of variation that are contained within the reported results. First, there is variation that arises due to the fitting algorithm and every \((x_c, y_c)\) has an associated standard error that arises from this fitting. Second, there is variation that arises due to the multiple fitting procedure on subsets of the available data. Recall that fitting a 2-dimensional functional form is an estimation procedure that is a far more complex algorithm than with a typical 1-dimensional form, with slightly more or slightly less data it is possible to obtain a different model fit or for the fitting algorithm not to converge. To determine the variations of model fit possible with the given data, minimally two models were fit for each data set collected. The models were created by progressively eliminating the low intensity levels, wherein the greatest noise was observed. Finally, there is variation that arises due to each WLL run which examined a different field in the slide.

As an example of the magnitude of these variations, the \((x_c, y_c)\) with standard error listed in brackets for AR Se WLL2 fitting 1 is \((531.24 (3.00), 460.96 (3.29))\) and fitting 2 is \((534.32\ldots\))
(1.43), 468.25 (1.89)), while WLL3 fitting 1 is (533.77 (2.69), 463.66 (2.93)) and fitting 2 is
(535.14 (1.24), 470.28 (1.60)). The within WLL laser variation (model fitting) can be larger than the average between WLL variation.

Additionally, the excitation and emission \((x_e \text{ and } y_c)\) are not independent variables (for example, emission cannot be greater than excitation) and there are boundary conditions imposed by the measurement technology. The data was very close to these boundaries in several cases.

The statistical tests were not satisfactory as none of them accounted for the variation properly. The AIC tests and extra sums of squares F-tests utilized all the data from the Gaussian surface, but the results were underpowered likely due to substantial differences in the variance-covariance parameters of each surface. The MANOVA comparisons, which are comparisons of the joint means, only use the observed variation in the mean. This difference in location was very small relative to the actual width of the respective Gaussian surfaces and the tests were overly sensitive. Due to these issues, we used visual comparisons among the treatment options via a scatter plot instead.
We believe ANOVA is a better choice for the intensity comparisons (Figs. 4C, 5C and 5D) because most of the groups have intensity values that appear normally distributed with approximately equal variances. The few groups that have much larger variances or distributions that might be either skewed or multimodal would cause just as many problems for the analogous nonparametric test as the one-way ANOVA, so we prefer to use the more sensitive ANOVA tests.

In all cases, these statistics face a continual challenge in that the sample size is inherently small. For each sample preparation, there are only 3 readings that were taken with each of the lasers. For the 405 and 458 nm emission data, the \( n = 3 \). For the WLL data, each data set was fit using OLP and the two fits using the greatest number of data points were collected. Hence, in this case, \( n = 6 \).
Figure S1
Figure S1 Output measurement at WLL pump power 100% vs. 60%

Changes in relative power output at different locations in the WLL output spectrum when the WLL pump power is set at 100% and 60% respectively. Blue bars (100% pump power) and red bars (60%).

Figure S2 (available at the Journal of Histochemistry and Cytochemistry website)
Figure S3 Additional examples of multi-lambda data fit to 2D Gaussian form

3D surface plots of raw multi-lambda data fit to the 2D Gaussian model. (A-B) AR Se additional fields and (C-D) EBT AR Se additional fields. 1230 pts is the full raw data set. 810 pts is next step in data point removal.
Comparison of green wavelength autofluorescence (AF) in tracheal FFPE tissue slides that received antigen retrieval and/or serum application: (A) AR Se, (B) No AR No Se, (C) AR No Se and (D) No AR Se. Images were taken on a Leica SP8 confocal system and are presented as maximum projections of 11.33 μm sized z-stacks; excitation was with an Argon
laser (488 nm) at 4% and detection range was 504-553 nm. White stars denote the tracheal lumens. Arrows denote red blood cells within vessels and extravasated red blood cells.

Bar: 20 μm.
Figure S5 Antigen retrieval and serum application fail to increase autofluorescence (additional data)

These 3D surface plots provide additional support that the model consistently fits the multi-lambda data: (A) No AR No Se, (B) No AR Se and (C) AR No Se. AR Se is shown in Fig. 3C. The range in variation of emission intensities visible within a treatment is consistent and overlapping with other treatments: (D) 3D surface plot of highest and lowest intensity value surfaces from all 4 treatments.
Figure S6 The effect of sodium borohydride is typically enhanced by AR and Se

3D surface plots of model fits to all raw data points for three fields from NB AR Se and NB No AR No Se compared to a representative AR Se demonstrate the effect of AR and Se when combined with NB treatment.
Figure S7 Treatments quench autofluorescence in frozen human liver

Eriochrome black T and Sudan black B have a similar effect on tissue autofluorescence in frozen cirrhotic human liver to that seen in formalin-fixed paraffin embedded human trachea.
Figure S8 shows the entire analysis process that was followed in this research. The process starts in the upper left hand corner with the creation of the treatment slides. Following the
arrows leads to two different scanning processes that were used to analyze the slides. For each of the scanning processes different assessment and evaluation techniques were employed. One scanning process created 2-dimensional data which was analyzed with line graphs and box plots of the maximum intensities. The other scanning process created 3-dimensional data. The scanning software itself produces several images and output arrays (the two right most arrows out of LAS AF A² scans) and the data were also analyzed by a mathematical software that allowed us to fit surfaces to the data to permit comparison of the different treatments. This allowed us to created 3-dimensional surface plots as well as assess and compare the location of the central peak and the relative intensity observed at this peak for each of the treatments. ANOVA analysis was used to compare the maximum intensities between treatments to determine is there were any significant differences. This is summarized in the connected letters reports.
CHAPTER 3

Validation of Normal Human Bronchial Epithelial Cells as a Model for Influenza A Infections in Human Distal Trachea
Abstract

Primary normal human bronchial/tracheal epithelial (NHBE) cells, derived from the distal-most aspect of the trachea at the bifurcation, have been used for a number of studies in respiratory research, including influenza A viral infections. Differences between the source tissue and the differentiated primary cells may impact infection studies based on this in vitro model. Therefore, we examined how well differentiated NHBE cells compared to their source tissue, human distal trachea, as well as the ramifications of these differences on influenza A viral pathogenesis research using this model. We employed histologic analysis including morphological measurements, electron microscopy, multi-label immunofluorescent confocal microscopy, lectin histochemistry, and microarray expression analysis to compare differentiated NHBEs to human distal tracheal epithelium. Pseudostratified epithelial height, cell type variety and distribution varied significantly. Electron microscopy confirmed differences in cellular attachment and paracellular junctions. Influenza receptor lectin histochemistry revealed that α2,3 sialic acids were rarely present on the apical aspect of the differentiated NHBE cells while this receptor was present in low numbers on distal trachea. Experiments in which fluorochrome bioconjugated virus was bound to respiratory tissue and NHBE cells or NHBE cells infected with human influenza A viruses, indicated that the pattern of infection progression in these cells correlates with autopsy studies of fatal cases from the 2009 pandemic.
Introduction

Normal human bronchial epithelial (NHBE) cells, also known as normal human tracheobronchial epithelial cells, a primary cell line derived from biopsies at the distal-most aspect of trachea and bronchial bifurcation, have been used for a number of studies in respiratory biology as well as respiratory disease research including therapeutics development (Hackett et al., 2009; Kesimer et al., 2009; LeSimple et al., 2010; Lopez-Souza et al., 2009; Thompson et al., 2006b; Triana-Baltzer et al., 2010; Zhang et al., 2005). Post-harvest and pre-distribution to researchers, these cells are de-differentiated by the vendor. Typically, researchers differentiate the cells into pseudostratified epithelium, composed of basal, ciliated and non-ciliated (goblet) cells, prior to conducting their experiments (Gray et al., 1996). Differentiated NHBEs have been compared to undifferentiated NHBEs and trachea, their source tissue, by microarray analysis (Dvorak et al., 2011; Martinez-Anton et al., 2013; Pezzulo et al., 2011). Additionally, their sialic acid receptor (Sia) distribution has been examined (Kogure et al., 2006; Thompson et al., 2006a). However, the findings reported are conflicting. These cells support influenza A virus infections (Brookes et al., 2011; Chan et al., 2010; Ilyushina et al., 2012a; Ilyushina et al., 2008; Ilyushina et al., 2012b; Mok et al., 2011; Oshansky et al., 2011; Scull et al., 2009; Song et al., 2009; Steel et al., 2008; Suksatu et al., 2009; Triana-Baltzer et al., 2010; Wan and Perez, 2007).

Despite this wide use, a side-by-side comparison of the morphology and cell type characteristics of these cells compared with distal trachea is lacking. Differences between the source tissue and the re-differentiated primary cell line may impact respiratory viral
infection studies based on this in vitro model. Therefore, this study examined how well differentiated NHBE cells compared to their source tissue, human distal trachea, as well as the ramifications of these differences on influenza A virus pathogenesis research conducted with differentiated NHBE cells.

In this study, we employed histologic analysis, immunofluorescence, and electron microscopy to characterize differentiated NHBEs in greater detail in comparison to their source tissue. Second, influenza receptor distribution in differentiated NHBE cells was compared to that in distal trachea and carina samples using lectin histochemistry for Sia (Nicholls et al., 2007; Suzuki, 2005). Third, we examined the binding pattern of Alexa Fluor-594 (AF594) succinimidyl ester carboxylic acid conjugated influenza A/NY/312/2001 (H1N1) virus (NY312) to sections of distal trachea, carina and differentiated NHBEs. Fourth, we conducted a series of infection studies with seasonal and 2009 pandemic influenza viruses, NY312 and A/CA/04/2009 (H1N1) virus (CA04) respectively. Finally, we evaluated influenza A virus antigen distribution in conjunction with apical cell type markers in a section of human trachea from an autopsy of an individual who died from a primary infection with 2009 pandemic influenza in tandem with the differentiated NHBEs infected with CA04.
Materials and Methods

Tissues

Anonymous donor distal tracheal tissue, with a post-mortem interval of several hours, was sourced from 2 cm proximal to the carina, as well as lung tissue, and was fixed in 10% neutral buffered formalin (NBF), dehydrated and embedded in paraffin (Capital Biosciences; Rockville, MD). Additionally, anonymous post-mortem tracheal and carina tissues were obtained from the Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, NIH (Bethesda, MD) and similarly processed. Immediately adjacent tissue samples from the latter were also fixed in 2.5% glutaraldehyde in 0.1M Sorensen's sodium phosphate buffer for electron microscopic analysis. This study also used formalin-fixed, paraffin embedded (FFPE) autopsy tissues as described by Gill et al. (Gill et al., 2010). Finally, additional anonymous normal human tissue sections used for immunohistochemistry controls were obtained from the Laboratory of Pathology.

Normal Human Bronchial Epithelial Cells

Primary Normal Human Bronchial/Tracheal Epithelial (NHBE) cells (CC-2541, Lonza; Walkersville, MD) from a single anonymous donor were differentiated following manufacturer’s instructions. Briefly, passage 1 NHBE cells were grown submerged in bronchial air-liquid interfaced (B-ALI) growth medium supplemented with Lonza SingleQuots (expansion media) in 6.5-mm transwell-clear membrane supports (inserts) coated with rat tail collagen (BD Bioscience; Bedford, MA) inserted in 24-well plates until they reached confluence (3-5 days) at which point the apical medium was removed in order
to create an air liquid interface and the basal compartment medium was refreshed with B-ALI differentiation media. During the 4 week long cellular differentiation period, medium in the basal compartment was changed every 48 hours and once mucus production commenced, mucus was removed at the same frequency using PBS washes and gentle aspiration technique. At 28 days, cell cultures reached mature pseudostratified epithelium status based on prior experience (Martinez-Anton et al., 2013) and direct microscopic examination and were considered to be ready for infections. Samples were fixed in NBF for light and confocal microscopy analyses and in 2.5% glutaraldehyde in 0.1M Sorensen's sodium phosphate buffer for electron microscopic analysis.

Viruses
All influenza viruses were propagated in Madin-Darby Canine Kidney epithelial cells prior to their use for bioconjugation or infection studies using standard technique (Balish et al., 2013). The following previously described viruses were used in this study: A/NY/312/2001 (NY312) and A/California/04/2009 (CA04) (Jagger et al., 2010; Qi et al., 2009). All viruses and infectious samples were handled in accordance with the guidelines of the National Institutes of Health under the supervision of the NIH Department of Health and Safety.

General Immunofluorescence Techniques

Five µm-thick sections of formalin-fixed, paraffin embedded cells or tissues were placed on positively charged slides (NC State University College of Veterinary Medicine Histopathology Lab; Raleigh, NC). Slides were deparaffinized, rehydrated and unless
otherwise stated, heat antigen retrieved in pH 6 citrate buffer (Dako-Target retrieval buffer s1699, DAKO, Carpinteria, CA) using a vegetable steamer technique for 15 minutes followed by 20 minutes cooling. Unless otherwise stated, tissues or cells were blocked prior to application of the primary antibody for 20 min with 5% normal serum, matched to the animal in which the paired secondary-fluorochrome conjugated antibody was raised, diluted in TBS. Prior to lectin application a streptavidin-biotin block (SP-2002, Vector Labs (VL); Burlingame, CA) and a carbohydrate block (SP-5040, VL) were applied per vendor instructions. Prior to application of fluorochrome bioconjugated lectin or virus a carbohydrate block was applied as above. Each reagent was applied separately and between each reagent application, 10 min washes with intermediate wash exchanges were conducted using TBS with 0.005 – 0.01% Tween-20 added. We used multiple Dylight conjugated Jackson Immunoresearch (West Grove, PA) secondary antibodies. These have been discontinued but equivalent fluorochrome conjugated secondary antibodies are available from this vendor and others.

Slides were imaged on both a Leica TCS SP5 X White Light Laser confocal system with Argon laser and 405 nm diode and a Leica TCS SP8 confocal system with 405 nm and 690 nm diodes and Argon, DPSS 561, HeNe 594 and HeNe 633 lasers. Both platforms were outfitted with a mix of Hybrid Detection (HyD) and photomultiplier tube (PMT) detectors and sequential image capture was almost always employed.
All multi-label protocols were built gradually with individual markers being validated independently then combined in a step-wise fashion with other markers. In each multi-label protocol run, we included a full set of single label slides so that any ‘cross talk’ between labels could be eliminated through laser and detector setting adjustments. During protocol development, we also applied the markers in a variety of orders to check for issues introduced by their order of application. Finally, we included control slides that received secondary antibodies or streptavidin conjugates but not the primary antibody or lectin. In each immunofluorescence methods section, markers are presented in the order in which they were applied on the final slides.

After application of all markers of interest, either DAPI (D1306, Molecular Probes (MP); Eugene, OR) at 0.3 µl per 10 ml TBS or DRAQ 5 (4084, Cell Signaling Technology; Danvers, MA) at 1:1000 in TBS were applied for 5 min, briefly washed then mounted in either Prolong Gold or Slow Fade Gold antifade reagents (P36930 and S36936, Life Technologies; Grand Island, NY). Finally, unless otherwise noted all primary antibody incubations were conducted for 1 hr at RT and all conjugated secondary antibody and streptavidin conjugate incubations were for 30 min at RT.

NY312 Alexa Fluor 594 Conjugate

Large batches of NY312 virus (12-T225 MDCK flasks) were β-Propriolactone (P5648, Sigma-Aldrich; Atlanta, GA) inactivated as previously described (Budowsky et al., 1993; Goldstein and Tauraso, 1970), concentrated by ultracentrifugation at 100,000g for 2 hr
at 4°C and purified using a 15/30/60% discontinuous sucrose gradient ultracentrifuged at the same settings with no brake. Each virus band was collected at the 30-60% interface, diluted 1:4 with sterile 1x PBS, ultracentrifuged again as in the initial concentration step, and the supernatant was collected for bioconjugation with Alexa Fluor 594 carboxylic acid, succinimidyl ester, mixed isomers (AF594-SE) (A-20004, MP) at a 4:1 virus/dye ratio. Virus (300 mg/ml), diluted to 500 ml in sterile PBS, was combined with 50 ml sodium bicarbonate pH 8.3 and 8 ml AF594-SE. The mixture was incubated for 1 hr on a stir plate at RT in the dark. The reaction product was transferred to a 100, 000 MWCO Vivaspin 500 centrifugal concentrator (VS0141, Vivaproducts; Littleton, MA) and centrifuged at 3700 g for 20 min in a benchtop mini centrifuge, yielding on average 200 ml of concentrated AF594-SE bioconjugated NY312 (NY312-AF594). Prior to inactivation, virus activity was confirmed by standard plaque assay (Gaush and Smith, 1968). After each stage in the process, the virus was tested to ensure maintenance of hemagglutinin binding capability using standard techniques (Balish et al., 2013). Protein concentrations were assayed using Bradford technique as per vendor’s instructions (23235 Micro BCA Protein Assay Kit, Pierce Biotechnology; Rockford, IL).

Jacalin Alexa Fluor 680 Conjugate

Unconjugated Jacalin lectin (L-1150, VL) was bioconjugated with Alexa Fluor 680 NHS ester (A-20172, MP) according to vendor kit instructions. Following the technique of Lantz and Holmes, 2001, the fluorochrome:protein (F/P) molar ratio (degree of labeling) was determined using the vendor provided correction factor of 0.05 and molar extinction
coefficient of Alexa Fluor 680 of 184,000 as well as the MW and extinction coefficient of Jacalin of 66 kDa and 1.5 respectively (Holmes et al., 2001). Absorbance measurements were made on a 10x PBS dilution of the conjugate on an Ultrospec II Spectrophotometer (Pharmacia LKB Biochrom, now Biochrom Ltd.; Cambridge, UK). The immunofluorescence efficacy of the AF 680 conjugated Jacalin was compared to biotinylated-Jacalin (B-1155, VL) visualized with streptavidin Alexa Fluor-680 conjugate (SAF-680) (S21378, MP). Each slide received a unique treatment as follows: the Jacalin-680 conjugate only (various concentrations ranging from 1:25 to 1:500 for 20 min), biotinylated Jacalin (5 µg/ml for 20 min) followed by SAF-680 (10 µg/ml for 30 min), or TBS only. DAPI was applied to all slides. Prior to its selection for bioconjugation, the specificity of the Jacalin for goblet cells was validated through a dual labeling of FFPE tracheal tissue with biotinylated Jacalin and Muc5Ac, a product of human goblet cells (Hovenberg et al., 1996). Briefly, biotinylated Jacalin was applied for 20 min at 2 µg/ml and visualized with streptavidin Alexa Fluor-488 conjugate (SAF-488) (S-11223, MP), Mucin 5Ac (MUC5AC)/Gastric Mucin Ab-1, Mouse Monoclonal Antibody (MA-145-P0, Thermo Scientific; Waltham, MA) was applied and visualized with Dylight 549-AffiniPure Donkey Anti-Mouse IgG (discontinued, Jackson Immunoresearch (JI)), and DRAQ5 for nuclei visualization. Normal human gastric tissue was used as a Mucin 5Ac positive control.

Microarray Expression Analysis

In order to ascertain whether neuroendocrine and professional immune cells were present in fully differentiated NHBE cells, we mined data from a previously completed microarray
analysis of a day 28, fully differentiated, NHBE cell sample from a non-smoker (Martinez-Anton et al., 2013). Briefly, sample preparation was completed according to standard Affymetrix procedures using the Genechip 3’IVT Express Kit and the Human Genome U133 plus 2.0 chip (Affymetrix; Santa Clara, CA). Specifically, we checked for the presence of the following genes CHGA, ENO2, SYP and NCAM1 expressed by neuroendocrine cells as well as CD68 expressed by monocytes, macrophages and dendritic cells, CD207 expressed by dendritic cells only and CD163 expressed by macrophages only. HMMR was a positive control. Data was generated using Affymetrix Expression Console Build 1.3.1.187. The probeset summarization was calculated using the MAS 5.0 signal intensity algorithm. Intensity values for each probeset along with their present absent calls were reported after running the Expression Console software on CEL files. Additionally, we referenced prior work when investigating the presence of club (Clara) cells (Martinez-Anton et al., 2013).

Immunofluorescence for Club Cells
Due to the presence in the differentiated NHBE microarray dataset of genes that encode proteins expressed by club cells, immunofluorescence for the presence of this cell type was done on FFPE differentiated NHBE cells, human lung and distal trachea without antigen retrieval using monoclonal mouse anti-human CC10 (sc-130411, Santa Cruz Biotechnology; Santa Cruz, CA) alone and in conjunction with the goblet cell marker, Jacalin. CC10 single labels on human lung, trachea and the cells were initially made using 1:50 dilution (dil) anti-CC10 paired with 1:400 Dylight 549-AffiniPure Donkey Anti-Mouse (discontinued, JI) and followed by DRAQ5. For the Jacalin and CC10 dual labels, biotinylated Jacalin (B-1155,
VL) was applied at 5 µg/ml for 20 min and visualized with Chromeo 494 streptavidin conjugate (16113, Active Motif; Carlsbad, CA) at 20 µg/ml; anti-CC10 was applied as above. When signal was absent on the NHBE cells and distal trachea at 1:50, antigen retrieval as previously described (Martinez-Anton et al., 2013) was added to the protocol and the antibody concentrations were increased to 1:25 overnight 4 °C (primary) and 1:200 (secondary), the maximum vendor recommended concentration, in a step-wise fashion. Further CC10 amplification using an intermediary biotinylation step with Biotin-SP-AffiniPure Donkey Anti-Mouse IgG (715-065-151, JI) was also tried.

Cell Type Marker Immunofluorescence on Differentiated NHBE Cells

Further image capture and analysis was conducted on differentiated NHBE cell type marker immunofluorescence work prepared for a prior publication (Martinez-Anton et al., 2013) and a new set of three wells of normal differentiated NHBE cells, grown at the same time as those used for the infection studies described later in this manuscript, was also labeled with cell type markers for this study. The methods applied to the new sections varied only slightly in choice of fluorochrome pairings from those used in the prior study. In brief, for slides created for the prior study, cells were detected with rabbit polyclonal anti-cytokeratin 5 (PRB-160P, Covance; Princeton, NJ) and visualized with Dylight 594-AffiniPure Donkey anti-rabbit IgG (711-515-152, JI); goblet cells were detected with biotinylated Jacalin and visualized with Chromeo 494 streptavidin conjugate; ciliated cells were detected with Beta-Tubulin Monoclonal Antibody, Mouse (2 28 33) (32-2600, Invitrogen; Carlsbad, CA) and visualized with Dylight 549-AffiniPure Donkey Anti-Mouse IgG, and nuclei were visualized with DRAQ5 (Martinez-Anton et al., 2013). For new cell samples labeled explicitly for this
study, cells were detected in the following order: basal cells as above, ciliated cells as above except that the prior fluorochromes conjugated secondary was replaced by Cy3-AffiniPure Donkey Anti-Mouse IgG (715-165-151, JI), goblet cells were detected with AF680-Jacalin (*de novo* conjugated as per above) at 1:50 dil for 20 min, and nuclei were visualized with DAPI. Finally, consequential to microarray results, we did single label immunofluorescence for Chromagranin A to screen for neuroendocrine cells in these samples as described in the next section where this marker is a component of a multi-label.

Cell Type Marker Immunofluorescence on Distal Trachea and Carina

In order to evaluate distribution of cell types of interest within the epithelium of the distal trachea and carina samples, a multi-label was constructed to visualize ciliated, goblet, basal, neuroendocrine and antigen presenting cells, macrophages and dendritic cells. The primary-secondary pairings were applied in the following order: macrophages and dendritic cells were detected with goat polyclonal anti-human CD68 Antibody (C-18) (sc-7082, Santa Cruz Biotechnology) at 1:25 dil and visualized with Cy3-AffiniPure Donkey Anti-Goat IgG (705-165-147, JI) at 1:200 dil; neuroendocrine cells were detected with rabbit polyclonal anti-human Chromagranin A (A0430, DAKO; Carpinteria, CA) at 1:300 dil overnight at 4º C and visualized with Dylight 594-AffiniPure Donkey Anti-Rabbit IgG at 1:200 dil; basal cells were detected with aforementioned rabbit polyclonal anti-cytokeratin 5 at 1:300 dil overnight at 4º C and visualized with Alexa Fluor 633 Goat Anti-Rabbit IgG (A-21070, Invitrogen) at 8 µg/ml; ciliated cells were detected with the same beta-tubulin antibody as above at 1:50 dil and visualized with Dylight 488-AffiniPure Donkey Anti-Mouse IgG (discontinued, JI) at
1:200; and goblet cells were detected and visualized by application of the AF680-Jac as above. DAPI was applied to the sections. Prior to the application of the basal cell marker, the tissue was blocked with 10% normal rabbit serum, AffiniPure Fab fragment Goat Anti-Rabbit IgG (111-007-003, JI) was applied at 1:10 dil for 1 hr to block the prior rabbit primary antibody, tissue sections were fixed in 4% paraformaldehyde for 3 min, and blocked with 5% normal goat serum.

The above protocol used two rabbit raised primary antibodies as well as goat-raised primary and secondary antibodies. In order to specifically label the individual targets when two of the primary antibodies were raised in the same host species, we adapted the double labeling with two primary antibodies technique from Jackson Immunoresearch, using recommended serum blocks and the appropriate unconjugated Fab fragment. We found that the addition of a paraformaldehyde fixation step strengthened the coverage of the Fab fragment block and was non-damaging to later marker applications. We validated the overall technique with control slides including single marker labeling of serial sections and reordering of the order of antibody application to find the most reliable ordering. In order to avoid non-specific binding issues attributable to the two goat raised antibodies, we ordered the protocol steps such that the use of the goat raised secondary came significantly later in the protocol sequence than the use of the goat raised primary.
Morphology Observations by Light and Electron Microscopy

Initial observations of morphological differences were made using standard hematoxylin and eosin (H&E) slides of the human distal trachea, carina and NHBE cells. We also examined un-embedded fixed cells on their membranes that were labeled similarly for a subset of the cell markers as described above. These samples were examined using bright field and confocal microscopy stepping through the full epithelial height of individual cells in order to ascertain that individual cells touched the membrane. Further analysis was conducted using differential interference contrast (DIC) overlays of merged fluorescence images. Briefly, the most representative slice of DIC imaging was selected for each z-stack and merged with max projected images of multi-label fluorescence. This enabled outlining of individual cells and determination of their type based on co-localization of a given cell type marker with the cell membrane as seen in the DIC image. This technique was used both to compare cell type distribution differences between the FFPE human respiratory tissue and the NHBE cells and to analyze receptor lectin distribution by cell type. Additionally, 30 epithelial height measurements from unique locations were taken and averaged for 8 samples of differentiated NHBEs, 2 samples of distal trachea and 1 of carina. Measurements were made using Imaris 7.6.3 (Bitplane; Zurich, Switzerland) using the ruler to measure from the basal lamina to the apical epithelial surface without including cilia projecting above the apical cell surfaces.

We also reviewed samples of both the human respiratory tissue and differentiated NHBE cells by transmission electron microscopy and the NHBE cells by scanning electron microscopy. For this work, glutaraldehyde fixed differentiated NHBE cells were excised
from the plastic frame supporting the cell membrane using a number 11 scalpel and were placed in flat embedding molds (Ted Pella, Inc.; Redding, CA) perpendicular to the cutting surface before polymerizing. Samples were processed as previously described (Howe et al., 2010) for transmission electron microscopy (TEM) with two changes. First, processing was done at approximately 150 W and 250 W in a BioWave Model microwave processor (Ted Pella, Inc.), rather than 80 W and 250 W in the Model 3451, and second Araldite resin (Structure Probe, Inc.; West Chester, PA) rather than Spurr’s was used for embedment. For scanning electron microscopy (SEM), samples were processed as described for TEM through post-fixation in osmium, and dehydrated in EtOH as described (i.e no UAc treatment). After dehydration in EtOH, the samples were critical point dried through liquid CO₂, lightly sputter coated with iridium, and examined on a model SU8000 field emission scanning electron microscope (Hitachi High Technologies America; Dallas, TX). Digital images were captured using the built-in frame grabber and an 80 sec scan. When we failed to visualize the rat-tail collagen coating on the NHBE cells membranes by TEM, we did immunofluorescence for collagen I using similar technique to that already described with rabbit polyclonal Anti-Collagen-I antibody (ab34710, Abcam; Cambridge, MA) at 1:50 dil overnight at 4º C and Dylight 549-AffiniPure Donkey Anti-Rabbit IgG at 1:200 dil (discontinued, JI).

Lectin Histochemistry with Apical Cell Type Markers

In order to examine the distribution by apical cell type of α2,3 versus α2,6 linked sialic acids at the apical aspect of the epithelium of the differentiated NHBEs as well as the human distal trachea and carina samples, lectin histochemistry was combined with the aforementioned cell
type markers for apical epithelial cells, goblet and ciliated cell types. For α2,3 linkages both MAA I and II, biotinylated *Maackia amurensis* isotype I and II lectins (B-1315 and B-1265, VL), and for α2,6, SNA, biotinylated mixed isotype *Sambucus nigra* lectin (B-1305, VL), were used. We used MAA II in conjunction with MAA I in order to examine both α2-3Galβ1-4GlcNAc as well as α2-3Galβ1-3GalNAc sialic acids (Nicholls et al., 2007; Suzuki, 2005). Since MAA I also has affinity for non-sialic acid moieties we interpreted its binding pattern in the context of a matched neuraminidase treated control. Goblet cells were detected with AF680-Jacalin. Ciliated cells were detected as before and visualized with Cy3-AffiniPure Donkey Anti-Mouse IgG. One receptor lectin per slide was detected with biotinylated MAA I or II at 20 µg/ml or SNA at 5 µg/ml for 20 min and visualized with SAF-594 (S-11227, MP) at 10 µg/ml. Nuclei were visualized with DAPI. A 90-min incubation at RT of 4UN/ml or 8UN/ml *Clostridium perfringens* neuraminidase (N2876, lot #071M8636V, Sigma-Aldrich, City, St), which strips the sialic acids from cells, served as an additional negative control, confirming the specificity of these lectins. The higher concentration was required to completely strip the sialic acids from the NHBE cells.

**NY312-AF594 Binding to Cells and Tissues**

A modification of the above protocol for cell type markers was also employed to label the apical epithelial cells in combination with bound AF594-NY312 on distal trachea, carina and differentiated NHBE cells. Initially, time and concentration titrations of bioconjugated virus with the addition of DAPI were conducted in order to determine a combination that yielded a consistent and reliable binding to targets of interest. Before each protocol run, the AF594-
NY312 hemagglutination-binding unit was determined using standard technique (Hirst, 1942) and the concentration adjusted accordingly in order to normalize between runs. In brief, markers were labeled as follows: goblet cells were detected with AF680-Jacalin as above; ciliated cells were detected with the anti-Beta-tubulin as in the lectin histochemistry section; appropriate concentrations of AF594-NY312 dil in TBS were applied for 30 min at RT; and nuclei were visualized with DAPI. Neuraminidase treated control slides were made to check for loss of viral binding in the absence of the sialic acid receptors for the virus.

Receptor Lectin and Virus Binding Competition
In order to confirm AF594-NY312 affinity for the same receptors as those labeled by receptor lectins, AF594-NY312 and biotinylated-SNA were applied as prior in alternating order on individual slides. Biotinylated SNA was applied at 5 µg/ml and detected with 10 µg/ml streptavidin Alexa Fluor 488 conjugate (S-11223, MP). SNA only and virus only control slides with and without neuraminidase pre-treatment were also included.

NHBE Influenza A Infection Studies
Uninfected control inserts were immersed in NBF prior to conducting infections on the cells. In order to examine variation in infections between seasonal and pandemic 2009 viruses, infections were conducted with both NY312 and CA04 at a multiplicity of infection (MOI) of 0.1 in triplicate (3 inserts per virus, time-point combination). Mucus was aspirated from each well, cells were incubated (37°C 5% CO₂) in PBS for 1 hr to solubilize remaining mucus, after which PBS was aspirated off. Virus at the appropriate dilution in 100 ul PBS
for the target MOI was applied, cells were incubated for 1 hr at 37°C 5% CO₂, virus was aspirated off the cells, cells were washed once with PBS, PBS aspirated, 100 µl of PBS was added to prevent dessication, and then cells were incubated until the appropriate end time point and placed in NBF. The time-points were 4, 8, 24, 48 and 72 hours.

Replication kinetics at these same time-points were determined for both viruses. Cells were infected with 100 µl of MOI 0.1 of virus, an additional 100 µl of PBS was added to prevent dessication, and cells were incubated for the appropriate number of hours. This experiment was also conducted in triplicate for each time-point, virus combination. At the appropriate times for each infected insert, apical supernatant was collected by adding 200 µl PBS to the sample, this fluid collected at the apical aspect, the wash repeated with an additional 200 µl PBS and the insert placed in NBF. All supernatants were stored individually in cryovials at -80°C. Standard plaque assays for influenza A with a crystal violet end product were done (Gaush and Smith, 1968). Graphs and analysis were done in Prism 6.0c (GraphPad; La Jolla, CA).

Immunofluorescence for Influenza Antigen with Cell Type Markers on NHBEs

Infected and uninfected (control) NHBE cells on their membranes were fixed in 10% neutral buffered formalin, processed and slides made as described above. After examination of multiple time-points for influenza antigen we selected the 24 hr time-point for demonstration of infected differentiated NHBE cells with cell type markers for both viruses. Influenza antigen only immunofluorescence used a 10% donkey serum block, primary antibody
polyclonal anti-H1N1 influenza A (ab20841, Abcam) at 1:400 was visualized with Dylight-594 AffiniPure Donkey Anti-Goat IgG (discontinued, JI) at 1:300. For the influenza antigen with cell type markers, ciliated cells were detected with the anti-β-tubulin antibody as above and visualized with Dylight 488-AffiniPure Donkey Anti-Mouse IgG (discontinued, JI) at 1:200. Goblet cells were detected and visualized with AF680-Jacalin. Influenza antigen was found as described above. Finally, basal cells were detected as before and visualized again with Alexa Fluor 633 Goat Anti-Rabbit IgG.

Immunofluorescence for Influenza Antigen with Cell Type Markers on Human Tracheal Tissue

Unstained positively charged slides of human tracheal tissue from a fatal 2009 pandemic influenza case lacking secondary bacterial infection discussed in Gill, 2009 was confirmed positive by influenza A viral antigen immunohistochemistry (Gill et al., 2010). Briefly, slides were prepared as for the immunofluorescence protocols above, including the same antigen retrieval. Tissues were blocked with 3% H2O2 dil in water for 10 min and Serum Free Protein Block (X0909, DAKO) for 30 min. The same anti-influenza primary antibody was used as per above and the LSAB+ System HRP (K0690, DAKO) was applied as per vendor instructions except that we extended incubation of both reagent steps to 25 min. Labeling was visualized with DAB Peroxidase (HRP) Substrate Kit, 3,3’-diaminobenzidine (SK-4100, VL) as per vendor instructions, slides counterstained with Mayer’s Hematoxylin and mounted in Permount Mounting Medium (17986, Electron Microscopy Services; Hatfield, PA). Normal uninfected tracheal tissue samples sourced from the Laboratory of Pathology
were used as uninfected controls. Consequently, sections of this tissue were treated identically to the infected NHBE cells described in the last section yielding sections immunofluorescently labeled for influenza antigen and cell type markers.

Confocal Image Capture and Analysis

For images that included a differential interference contrast overlay, the most representative slice of differential interference contrast imaging was selected for each z-stack and merged with max projected images of multi-label fluorescence. All image post-processing and analysis was done with Imaris then Adobe Photoshop CS6 (Adobe; San Jose, CA). Where statements regarding relative intensity are made, e.g. receptor lectins, influenza antigen, or bioconjugated virus, the signal intensity levels for the markers were kept constant during image capture and post processing. Final figure compilation was done in Adobe InDesign CS6 Extended (Adobe).
Results
Comparison of Normal Human Bronchial Epithelial Cells to Their Source Tissue Type
The cell types present in the differentiated NHBE pseudostratified epithelial layer were a subset of those identified in the pseudostratified epithelial cell layers of the distal trachea and carina tissue. Jacalin-680 consistently identified goblet cells. Jacalin co-localized with Muc5Ac on distal trachea demonstrating that Jacalin is also specific for mucin producing goblet cells (Fig. S1). At the apical epithelial aspect, multi-label immunofluorescence for cell type markers revealed β-tubulin positive ciliated cells and Jacalin positive non-ciliated cells. Non-apical, round, cytokeratin-5 positive cells, interpreted as basal respiratory epithelial cells, were present at the basal epithelial aspect (Fig. 1, S2-S5). Distal trachea and carina epithelia additionally contained pyramidal-shaped, Chromagranin A positive cells, interpreted as neuroendocrine cells, and CD68 positive cells morphologically consistent with macrophages and dendritic cells. All signals were cytoplasmic, consistent with expectations for these markers. These findings were also consistent with those predicted by the microarray expression analysis.

Neuroendocrine, professional immune and club (Clara) cells were absent from differentiated Normal Human Bronchial Epithelial cells (NHBEs). Expression of the following genes was absent in the differentiated NHBE cells: CHGA, SYP, NCAM1, CD68, CD207, CD163. Expression of ENO2 and HMMR was present (Table). Since club cell specific genes SCGB1A1 and CYP2B6 were increased in differentiated NHBEs (Martinez-Anton et al., 2013), we sought club cells using immunofluorescence. The anti-CC10 IF yielded a
consistent cytoplasmic signal from cells in the positive control human lung tissue at the expected locations from cells that were morphologically consistent with club cells. These cells were most abundant at the level of the respiratory and terminal bronchioles with lower numbers in the larger bronchi. However, differentiated NHBE cells and human distal trachea were consistently negative despite amplification attempts. Finally, we saw no cells morphologically consistent with these cell types in undifferentiated or differentiated NHBE cell cultures using both light microscopy and Differential Interference Contrast (DIC) microscopy.

Fully differentiated NHBEs appeared to consistently form a true pseudostratified epithelium morphologically similar to that of carina and distal trachea but different in height and cell type distribution. All cells traced in NHBE layers reached the membrane just as cells in distal trachea and carina contacted the basement membrane. The NHBEs consistently have a thinner pseudostratified epithelium than carina or distal trachea tissue. The average epithelial heights for the differentiated NHBEs, distal trachea and carina were 16.5, 50.8, and 40.9 mm respectively. The NHBE measurements were significantly different from the distal trachea and carina by One-Way ANOVA (Fig. S6).

Transmission electron microscopy revealed cells consistent with ciliated, goblet (mucous) and basal cells were present in both the NHBEs and source tissue as expected (Fig. 2E-F) and tight junctional collars composed of desmosomes were also seen at the subapical aspect (Fig. 2G-H). However, NHBE cells consistently demonstrated increased laxity in paracellular
junctions, both between basal cells and between basal cells and the other epithelial cells, when compared to distal trachea and carina (Fig. 2I-J). Additionally, cellular attachment to the artificial membrane by the NHBEs was limited to an occasional lamellipodium of a basal cell interacting with a membrane pore (Fig. 2K). In contrast, the distal trachea and carina samples demonstrated both basement membrane substance and hemidesmosomes (Fig. 2L). The cilia density appeared to be lower on both individual ciliated cell surfaces and on the whole epithelial surface. The β-tubulin labeling of NHBEs (Fig. 1), review of unembedded membranes by light microscopy and scanning electron microscopy (Fig. 2, Row 1) revealed this frequently sparse distribution of ciliated cells. Non-ciliated cells dominated the apical aspect of the epithelium. Even despite the poorer preservation state of the carina and distal trachea EM samples this cilia density difference was maintained at the EM level (Fig. 2E-F).

Comparison of Receptor Lectin Histochemistry and Fluorochrome Conjugated Influenza Binding

Clostridium perfringens neuramidase effectively removed sialic acid receptors (Sia) preventing Sambucus nigra (SNA) and Maackia amurensis II (MAA II) from binding either the human respiratory tissue or NHBE cells (Fig. S7). In contrast, post neuraminidase (NA) treatment, the extent of labeling with MAA I overall increased on all targets (Fig. S16). Interestingly, NHBEs required twice the concentration of neuraminidase as compared to the human tissue samples to successfully remove all the Sias.
SNA consistently labeled the entire apical epithelial aspect of the NHBE cells (Fig. 3A). Combining its signal with that of cell type markers for the ciliated and goblet cells revealed that it labeled both cell types (Fig. 3B, S8). A similar pattern was seen with SNA in distal trachea (Fig. 3C-D, S9-11). MAA II labeled rare apical aspects of NHBE cells. When these cells were examined in conjunction with cell type markers they were consistently goblet cells (Fig. 3E-F, S12). On distal trachea and carina sections, MAA II labeled more cells overall, both the apical aspect of some and the mucins within goblet cells (Fig. 3G-H). In these tissues, there was also some MAA II signal correlation with ciliated cells, particularly in the first trachea sample (Fig. S13). However, this correlation was not observed in the second trachea sample and the carina (Fig. S14-15). On NHBE cells untreated with NA, MAA I labeled ciliated cells (Fig. S16A-B). In contrast, post NA treatment, MAA I labeled nearly the entire apical epithelial surface, corresponding to a mix of ciliated and goblet cells (Fig. S16C-D). On distal trachea, MAA I faintly labeled nearly the entire apical epithelial surface as well as submucosal gland epithelial cells (Fig. S16E-F). Again, as with the NHBE cells, the MAA I labeling intensity increased post NA treatment and included goblet cell mucins (Fig. S16G-H).

The binding pattern for bioconjugated influenza virus, NY312-AF594, to NHBEs was similar to that observed for human tissue. The bioconjugated virus bound to the apical aspect of both ciliated and goblet cells as well as the mucins within the goblet cells that had been exposed by sample sectioning (Fig. 4). Individual marker images, merges, DIC and fluorescence merges with DIC overlay images are shown in (Figs. S17-18). Pre-treatment of
tissues with neuraminidase practically eliminated the NY312-AF594 that bound the NHBE cells and tissues (data not shown). Additionally, in order to confirm that SNA and the NY312-AF594 competed for the same receptors we labeled NHBE cells and distal tracheal tissue with SNA and NY312-AF594 in alternating order. When the bioconjugated virus was bound first the level of SNA that bound second was less (Fig. S19). Conversely when the SNA was bound first the level of virus bound second was decreased.

Comparison of Pandemic 2009 virus infections of NHBEs to 2009 Pandemic Autopsy Tissue Histopathology of H&E-stained NHBE sections for the 4, 8, 24, 48, and 72-hour time-points was similar for the NY312 and CA04 infections. At 4 hours post-infection cells were still within normal limits (not shown). At 8 hours, there was increased cytoplasmic vacuolation within apical epithelial cells multifocally and increased paracellular lucency between cells (Fig. 5A). At 24 hours post-infection, this paracellular lucency increased, and the apical surface of the epithelial layer became uneven, interpreted as cell loss (Fig. 5B). Occasional pyknotic nuclei as well as apoptotic cell bodies were seen. At 48 hours, the cell layer was markedly attenuated where still attached (Fig. 5C). This pattern increased by 72 hours when frequently few cells remained attached to the membrane (Fig. 5D). These detached cell clumps did not always have visible cytopathologic changes. The replication kinetics showed that the virus titers for NY312 and CA04 were similar at matched time points (Fig. 5E).

We also compared influenza antigen and cell type (ciliated, goblet and basal cell) co-localization at the 24- and 48-hour post-infection time-points for the CA04 infected
NHBEs to human trachea from an autopsy case in which the patient died from a pandemic 2009 influenza infection (Gill et al., 2010). At the 24-hour post-infection time-point, influenza antigen positive goblet and ciliated cells were observed (Fig. 6, S20). Both cytoplasmic and nuclear labeling was present, Due to a small amount of non-specific background labeling not all the cytoplasmic signal could be interpreted as influenza antigen but the majority could (Fig. S21). A similar influenza antigen-labeling pattern was also present at the 48-hour post-infection time-point. However, by that time-point, labeling of NHBE basal cells for influenza antigen including their nuclei was also seen. The basal cells in the autopsy tracheal tissue were rarely positive for influenza antigen (Fig. 6, S22). The 72-hour post-infection time-point cells could not be examined by immunofluorescence due to frequent cell loss from slides during protocols.
Discussion

Overall the NHBE model is a useful *in vitro* option for modeling influenza A virus infections, particularly at early infection time-points. Since it is a primary cell line, differentiable into a pseudostratified epithelium, it facilitates close inspection of virus-epithelial cell interactions in a manner better correlated with three-dimensional *in vivo* reality. In this manner and because it is derived from normal, non-cancerous human cells, the NHBE model is a better choice than immortalized carcinoma-derived lung cell lines currently in wide use in influenza research such as A549s. Our results show that the epithelial cell types and their distribution resembled that of source tissue samples with a few differences. There was formation of a pseudostratified epithelium complete with apical tight junctional collar formation. Influenza A viral receptor lectin distribution patterns fairly closely resembled those of source tissue and interaction with influenza virus is similar. However, our observations raised concerns regarding the integrity of NHBE systems at later time-points in infection studies with pathogenic viruses. These issues appear to be due to differences in the basal aspects of the NHBE pseudostratified epithelial architecture. Future work should examine methods of strengthening the paracellular interactions and basal attachment of NHBE cells so that they can more closely model infections at later time-points. Such *in vitro* system enhancement might also encourage fuller development of the pseudostratified epithelium, seen as both increases in height and ciliated surface density.

Mature trachea and carina epithelium contained neuroendocrine cells as well as occasional CD68 positive cells, interpreted as macrophages and dendritic cells. Our NHBE microarray
data analysis results were generally consistent with an absence of both neuroendocrine cells and professional immune cells, specifically macrophages and dendritic cells in the differentiated NHBE sample analyzed. The presence of ENO2 (g-enolase, neuron specific enolase) expression was surprising. Since it is associated specifically with neuron/neuroendocrine tissues (Ho et al., 2010), we could not rule out the presence of neuroendocrine progenitor cells. However the absence of CHGA, SYP and NCAM1 transcripts in our microarray data, the lack of pyramidal shaped cells morphologically consistent with mature neuroendocrine cells and negative immunofluorescence for chromagranin-A all suggest that mature neuroendocrine cells were absent. Additionally, we saw no dendritic cells and macrophages. Confirmation of the absence of these cell types is significant to downstream cytokine analysis of interest in influenza experimental studies focused on host response to infection. Our findings were also consistent with the morphological reviews of these cells by LONZA during their quality control process (vendor communication). Extensive flow cytometric analysis of NHBE cells might reveal further information but this was not conducted as part of this study.

Since we had previously observed up-regulation of genes that localize to club (Clara) cells, specifically multiple secretoglobins (club cell secretory proteins) and CYP2B6 (a member of the cytochrome p450 family) in the differentiated NHBEs when compared to the undifferentiated ones, we conducted further investigations seeking club cells in the differentiated NHBEs (Hukkanen et al., 2002; Martinez-Anton et al., 2013; Mori et al., 1996; Wong et al., 2009). Our immunofluorescence for anti-CC10 was consistently negative
on our FFPE differentiated NHBE cells and the human distal trachea samples. In contrast, the FFPE human lung tissue consistently had positive cells. Histologically, we observed no cells that were morphologically consistent with club cells (non-ciliated, dome-shaped but non-mucin producing) (Pack et al., 1981). By TEM, we saw variation in the intracytoplasmic granule morphology and cytoplasmic electron lucency of non-ciliated cells (Fig. 2E). Club cell secretory granules reportedly contain abundant endoplasmic reticulum and are smaller in number and size than those of mucous cells (Bedetti et al., 1987; Gruenert et al., 1995). We found no cells that were clear morphologic matches. The occasional non-ciliated morphologically divergent cells may have been less common “brush cells,” cholinergic chemosensory cells, pre-ciliated or other less well-defined cell types (Brand-Saberi and Schafer, 2014). Immunoelectron microscopy techniques might provide further clarity. Therefore, while the transcripts for multiple club cell related proteins were present, protein products were not detectable by immunofluorescence assay and morphologic support was lacking. Perhaps our cell culture microenvironment failed to support full differentiation club cells. Regardless, due to these findings NHBEs are consistent with their source tissue because club cells are rarely found above the level of the terminal bronchioles in humans and absent in their bronchi (Boers et al., 1999).

Jacalin, Artocarpus integrifolia lectin, isolated from jackfruit seeds is specific for O-glycosidically linked oligosaccharides (Lee et al., 2013). It has been used before to identify goblet cells in both NHBE cells and tracheal tissue where we and others have shown it to label equivalently to Muc5Ac in these cells (Fig. S1) (Chandrasekaran et al., 2008; Martinez-
Anton et al., 2013; Vermeer et al., 2003). Our selection of ciliated cell and basal cell markers was also driven by prior research (Ayora-Talavera et al., 2009; Martinez-Anton et al., 2013; Shelton et al., 2011; Thompson et al., 2006a). Like many, we chose a β-tubulin to label ciliated cells (REFs). We used cytokeratin-5 (CK5) in this and a prior study, but have also explored the use of cytokeratin-14 (CK14) and both CK5 and CK14 in conjunction with cytokeratin 8 (CK8), a luminal cell marker. In our hands CK5 more consistently labeled all the cells at the basal aspect of the NHBE epithelium that have the round, non-apical cell morphology and are also CK8 negative, consistent with a basal cell (data not shown). Together the markers we chose for goblet, ciliated and basal cells worked well in combination with lectins, antibodies and virus enabling cell type localization of each.

As in our prior study (Martinez-Anton et al., 2013), we found that the NHBE cells formed a pseudostratified epithelium by day 28-post confluence. Here, we determined that NHBE epithelial height was on average significantly shorter than that of the distal trachea and carina tissues (Fig. S6). Additionally the lawn of cilia produced by NHBE ciliated cells failed to cover the apical aspect of the epithelium as it consistently does in well-preserved source tissue sections (Fig. 1). Whether this is due to failure of the pseudostratified epithelium to fully develop or greater development of the goblet cells in respects to the ciliated cells is open to debate. However, the shorter NHBE epithelium height, decreased cilia density overall, and presence of a larger apical surface area of goblet cells, appreciable by both light and electron microscopy suggests the former (Fig. S6, 1, 2A-C, 2E-F) (Arenberg, 1972; Menscher, 2013; Roessler et al., 1991). Our findings also correlate with microarray
evidence that ciliated cell density is less in air-liquid cultures such as the NHBE system compared to large airway bronchoscopy brushings from healthy non-smokers (Dvorak et al., 2011). NHBE cilia appeared to be morphologically normal in size and structure on EM but were subjectively less dense in number per cell (Fig. 2E-H). This is something that might be assessed further in a study wherein the microenvironment was manipulated in order to enhance further development of the pseudostratified epithelium. The apical tight junctional collar of the NHBE epithelium resembled that of the source tissue (Fig. 2G-H) including presence of desmosomes as seen previously (Chopra et al., 1987). This validates the measurement of resistance during manipulation of NHBE cells to determine when the cell layer is compromised (LeSimple et al., 2010).

It was at the basal aspect of the NHBE epithelia that our findings were most divergent from source tissue. Compared with source tissue samples, we had observed increased lucency between basal cells and the basal aspects of apical cells, goblet and ciliated, in H&E slides of the NHBEs. On TEM, the differences in the spacing of these paracellular interactions were more apparent with significant clear space between cells. Paracellular contact between cells was less common in the NHBE samples as compared to source tissue samples (Fig. 2I-J). We observed the usual basal lamina formation and hemidesmosomal (fibrillar adhesion) attachment of the carina and distal tracheal epithelium to the submucosa. In contrast, inspection of the same location in the NHBE cultures revealed little to no attachment for the NHBE cells. On TEM, we found no evidence of the rat-tail collagen that had been applied to the membranes. Our immunofluorescence labeling for anti-collagen I on NHBE samples was
also negative. Interestingly, lammelapodia of NHBE cells were frequently observed inside membrane pores (Fig. 2I). These findings demonstrated that minimally the basal plasma membranes of some NHBE cells were forming focal complexes with extracellular matrix or membrane substance (Berrier and Yamada, 2007; Hermann et al., 2014).

As evidenced in this study (Fig. 5) and many others (Brookes et al., 2011; Chan et al., 2010; Ilyushina et al., 2012a; Ilyushina et al., 2008; Ilyushina et al., 2012b; Mok et al., 2011; Oshansky et al., 2011; Scull et al., 2009; Song et al., 2009; Steel et al., 2008; Suksatu et al., 2009; Triana-Baltzer et al., 2010), NHBE cells support human influenza A infections. Early stages of NHBE infections closely parallel the infection pattern seen in autopsy material (Fig. 6). However at later infection time-points, NHBE cell loss can be rapid and these detached cells can lack morphologic cell changes consistent with cell damage or death. Our observations regarding the minimal basilar attachment of differentiated NHBE epithelia may explain the presence of otherwise normal looking detached individual cells and clumps of cells intermixed with obviously affected cells that showed signs of reversible cell damage and cell death.

Limitations in epithelial integrity attributable to their in vitro microenvironment would be a concern when cell layer loss is used as a metric in a study. If they were more firmly attached, the NHBE cells might recover from infection. Closer examination of these later infection time-points will likely reveal that cell loss is due to both infection and stability of epithelial adhesions to the membrane. Separation of influenza A virulence-associated
changes in epithelial cell adhesion from *in vitro* system limitations might be provided by better understanding the status of focal complex and focal adhesion formation in the NHBE system (Berrier and Yamada, 2007). Similar work assessing the role of *Staphylococcus aureus* hemolysin A in primary and immortalized human airway epithelia revealed that it induces alteration in cell shape, formation of paracellular gaps, and prevented maturation of focal complexes into focal adhesions (Hermann et al., 2014). Perhaps research progress in human skin equivalents wherein pro-collagen type 1 secretion by human fibroblasts co-cultured with epithelial cells outperforms rat-tail collagen-based systems might inform improvement of the NHBE system (El Ghalbzouri et al., 2009; Kamolz et al., 2005). A co-culture system of NHBEs with normal human lung fibroblasts was previously used to examine basal cell and subepithelial matrix homeostasis (Thompson et al., 2006b). Alternatively, changing the preparation of the membranes might encourage better adhesion of the NHBE cells, enabling attainment of greater epithelial height and further cellular differentiation particularly of ciliated cells. Options include Collagen IV, more focused on attachment, instead of Collagen I, more focused on epithelial-to-mesenchymal transition, and wound healing (Hackett and Knight, 2007) and a substance such as Matrigel Matrix.

We examined our tissues and cells for \( \alpha_2,3 \) linkage N-acetylneuraminic (sialic) acids with *Maackia amurensis* and for \( \alpha_2,6 \) linkages with *Sambucus nigra* (SNA) (Chan et al., 2013; Nicholls et al., 2007). *Maackia amurensis* leukoagglutinin (MAA I or MAL) preferably binds Sia\( \alpha_2-3 \)Gal\( \beta_1-4 \)GlcNAc in N-linked or core 2 O-linked sialic acids as well as unsialylated glycan, SO\( _3-3 \)Gal\( \beta_1-4 \)GlcNAc (Geisler and Jarvis, 2011). *Maackia*
*amurensis* hemagglutinin (MAA II and MAH) preferably binds Sia\(\alpha 2-3\)Gal\(\beta 1-3(\text{+Sia}\alpha 2-6)\)GalNAc in O-linked sialic acids as well as SO\(_4\)-3-Gal\(\beta 1-4\)GlcNAc (Geisler and Jarvis, 2011). Data from the Consortium for Funcational Glycomics’ (http://www.functionalglycomics.org/) binding of these lectins to defined glycan arrays confirmed these binding specificities and additionally revealed that MAA I can bind \(\alpha 2,8\) linkage N-acetylneuraminic (Sialic) acids and that MAA II can bind all structures containing SO\(_4\)-3-Gal\(\beta\) (Geisler and Jarvis, 2011). Therefore, we avoided using *Maackia amurensis* lectins from vendors where these two isoforms are mixed together in undefined ratios. We also selected a neuraminidase that is purportedly efficacious at removing \(\alpha 2,3,\alpha 2,6,\) and \(\alpha 2,8\) linkage N-acetylneuraminic (Sialic) acids. In retrospect, additional use of an \(\alpha 2,3\) specific sialidase may have eased data interpretation. However the *Clostridium perfringens* neuramindase that we used cleaves \(\alpha 2,3\) sialic acids most efficiently.

Our findings were similar to others in that the NHBE cells, trachea and carina labeled predominantly with SNA and MAA I and rarely with MAA II (Fig. 3, S16) (Chan et al., 2013). We attributed the post neuraminidase (NA) treatment MAA I signal to the non-sialic acid moieties that this lectin also binds (Fig. S16) (Geisler and Jarvis, 2011; Nicholls et al., 2007). However, we were surprised by the overall signal intensity increase post NA treatment. Prior lectin blotting work has shown a reduction in MAA I and elimination of MAA II signal post \(\alpha 2,3\) sialidase treatment (Geisler and Jarvis, 2011). Perhaps the action of the NA resulted in direct exposure of additional Gal\(\beta 1-4\)GlcNAc, which is a secondary target.
of MAA I but not MAA II (Geisler and Jarvis, 2011). We also concluded that our targets were not SO₄-3-Galβ1-4GlcNAc rich due to the lack of the post-NA treatment binding of MAA II. In summary, both our cells and tissues displayed predominantly α2,6 linkage sialic acids.

Only signal present at the apical aspect of an epithelial cell with luminal presence represents a sialic acid that might function as an influenza receptor. Therefore, we discounted signals internal to cells or from cells lacking contact with the lumen. Reports in the literature for sialic acid distribution by cell type in NHBE cells vary. They include α2,3 linkage primarily expressed on ciliated cells (Matrosovich et al., 2004), α2,6 on both ciliated and goblet cells with no presence of α2,3 O-linkage sialic acids (Chan et al., 2010; Ibricevic et al., 2006), and dual expression of both linkage types on the individual cells (Kogure et al., 2006). Our co-labeling with sialic acid receptor lectins and apical epithelial cell type markers revealed that there was rare labeling of NHBE goblet cells with MAA II, contrary to the sparse labeling of ciliated cells reported with this lectin prior (Fig 3, S12) (Chan et al., 2010). This co-localization of Jacalin with MAA II correlates well with both lectins’ affinity for O-linked glycans. MAA I labeled ciliated cells predominantly (Fig. S16) while SNA labeled both goblet and ciliated cells (Fig 3, S8). Use of Concanavalin, a lectin with affinity for N-linked glycans, might be used as a confirmatory step in future work (de Graaf and Fouchier, 2014).
We found that our trachea and carina samples either rarely labeled with MAA II (Fig. S14-15) or labeled low numbers of goblet cells as well as cross sections of some goblet cells (Fig. S13). The latter is consistent with mucus being α2,3 sialic acid rich as previously reported (Baum and Paulson, 1990). Interestingly, the first trachea sample also had some mucous globules that were strongly SNA positive (Fig. S9). SNA labeling otherwise matched that seen for the NHBE cells (Fig. S9-11). MAA I labeling of trachea was faint but relatively widespread across both cell types (Fig. S16). Perhaps this correlates with a residual mucus layer. Therefore, in general, differentiated NHBE cells displayed influenza receptor lectins similarly to their source tissues.

Since NY312 has an α2,6 sialic acid binding preference, the NY-312-AF594 virus binding provided a confirmatory assay for the SNA binding. Results were as predicted with SNA and NY312-AF594 competing for the same receptors (Fig. 3, 4, S19). Fluorochrome conjugated viruses have been used in influenza research before (Brandenburg and Zhuang, 2007; Couceiro et al., 1993; Yoshimura and Ohnishi, 1984). In particular, as prepared here they would be useful for comparisons of influenza virus receptor binding domain variants’ binding patterns. This might include competitive binding between two viruses if each virus was bioconjugated with a different fluorochrome, examination of binding and entry in live cell imaging studies and examination of the early stages in the viral life cycle via supra-resolution microscopy.
Histologic changes attributable to influenza infection were visible in apical cells as early as the 8 hr post infection time-point (Fig 5). At 24 hr post infection, infected NHBE cells demonstrated similar influenza antigen distribution patterns to those found in autopsy tissues. Both apical cell types, ciliated and goblet, had nuclear and cytoplasmic influenza antigen labeling (Fig. 6). This pattern commenced at the 8 hr time point and increased steadily over time. These patterns were also similar to those we have seen in many mouse and ferret studies.

At 48 and more so at 72 hr post infection, NHBE basal cells were also infected. Additionally, many individual and cell clumps were detached from the membrane. We attributed the absence of cytologic signs of pathology in many of these cells to potential psuedostratified epithelia integrity issues (see above). Additionally, the abundance of basal cell influenza antigen positive cells at these later time point may be artificially increased due to these same issues.

Despite the extensive literature available on NHBEs including their use for influenza A research, we believe that our work adds value in its side-by-side comparison of NHBEs to their source tissue, including cell type presence and distribution, epithelial morphology comparison, influenza A receptor distribution, viral binding and infection pattern comparisons. Our study highlights both strengths and limitations of the NHBE model for influenza research and identifies potential methods for addressing some of these limitations.
Many of these findings translate to use of this model for other tracheobronchial mucosal infection studies and more general respiratory research.
Acknowledgements

I thank my co-authors on the manuscript based on this work that is currently under review: Daniel Chertow, Jenna E. Moyer, Jon Suzich, Aline Sandouk, David Dorward, Carolea Logun, James H. Shelhamer and Jeffery K. Taubenberger. The authors would like to acknowledge Larry Lantz of the NIAID Research Technology Branch (RTB) Custom Antibodies Section, NIAID for his assistance in the characterization of Jacalin-680 and the conjugation of influenza viruses with AF594 SE as well as the staff at the NIAID RTB Bioimaging Section for their assistance with confocal microscopy and image post-processing. Additionally, we would like to thank Raymond J. Pickles, University of North Carolina for thoughtful discussion about the labeling differences between anti-alpha- and anti-beta-tubulins. We thank Louis Schwartzman, VPES, LID, NIAID for his assistance with batch production of NY312 virus. We thank Sandra Horton and her staff at the North Carolina State University College of Veterinary Medicine Histopathology Laboratory for their sectioning of tissue and embedded NHBE cell sections for immunofluorescence and histological analysis. This work was supported by the intramural grant of NIAID, NIH. ASD and JKT are further thankful for the support of the NIH Comparative Molecular Pathology Research Training Program. This work was done in partial fulfillment of A. Sally Davis’ dissertation work towards a PhD in Comparative Biomedical Sciences at the North Carolina State University College of Veterinary Medicine.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Cell type</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHGA</td>
<td>Neuroendocrine</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>ENO2</td>
<td>Neuroendocrine</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>SYP</td>
<td>Neuroendocrine</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>NCAM1</td>
<td>Neuroendocrine</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>CD68</td>
<td>Macrophage/Dendritic Cell</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>HMMR</td>
<td>Positive control</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>CD207</td>
<td>Dendritic Cell</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>CD163</td>
<td>Macrophage</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>CD163</td>
<td>Macrophage</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

* Present (P) and absent (A)
Figure 1 Normal Human Bronchial Epithelial cell types compared to human distal trachea and carina

Leica SP8 laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded cells and tissues. All imaging was done with a 40x oil objective at zoom 2.7, 2048 x 2048 pixels. (Row 1, A-C) is differentiated Normal Human Bronchial Epithelial (NHBE) cells, (Row 2, D-F) human distal tracheal tissue, and Row 3 (G-I) human carina. (Col 1) Cell type markers including b-tubulin labeled ciliated cells (red), Jacalin labeled goblet cells (magenta), cytokeratin-5 labeled basal cells (green), nuclei (gray), neuroendocrine cells (blue) and CD68+ cells (macrophages/dendritic cells) (cyan); the last two markers were only done on the human tissues. (Col 2) Differential interference contrast (DIC), a representative single 0.20 mm thick slice showing cell outlines. (Col 3) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar is 10 µm.
Figure 2 Electron microscopy of NHBEs and human carina

(Row 1, A-D) Scanning electron micrographs of progressively higher resolution demonstrating the distribution of ciliated (c) and goblet (g) cells at the epithelial surface of fully differentiated normal bronchial epithelial cells. Transmission electron micrographs of fully differentiated Normal Human Bronchial Epithelial cells (NHBEs) alternating with human carina tissue (Rows 2, E-H, and 3, I-L). Magnification is marked with individual rulers at the bottom right of each image. (E) The differentiated NHBE cells, full thickness from membrane (m) to lumen (l); examples of ciliated (c), goblet (g), and basal (b) cells are annotated. The (*) denotes an atypical non-ciliated cell. Bar is 2 µm. (F) The full carina epithelial thickness with submucosa (s) and basement membrane denoted by arrows; examples of ciliated (c), goblet (g), and basal (b) cells are annotated. Bar is 2 µm. (G) A goblet cell between two ciliated cells with cilium basal body marked by white (*) and arrows denoting desmosomes, tight junctions, present between these cells. Bar is 500 nm. (H) A ciliated cell (left) next to a goblet cell (right) with arrows denoting tight junctions between them at their apical aspects; (*) marks cilium basal body. Bar is 500 nm. (I) Basal aspect of epithelium demonstrating laxity in the interdigitations between cells, white (*)s. White and black arrows denote a membrane pore with an adjacent basal cell’s lamellipodium-like extension within it (b). Bar is 500 nm. (J) Within the basal aspect of epithelium, paracellular junctions between epithelial cells, denoted by arrows, were tighter in the carina and distal tracheal epithelium compared with the NHBEs (J). Bar is 500 nm. (K) The basal aspect of epithelium, arrows, was not firmly attached to the membrane (m). Bar is 100 nm. (L) Arrows show that the basement membrane of the epithelium (e), was tightly adherent to the submucosa (s) by hemidesmosomes. Bar is 100 nm.
Figure 3 Influenza receptor lectin distributions in NHBEs and distal trachea

Leica SP5 white light laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded cells and tissues. All imaging was done with a 40x oil objective at zoom 2.0, 2048 x 2048 pixels. (Row 1, A-D) *Sambucus nigra* labeling of differentiated NHBEs and distal trachea, lectin labeling alone and then lectin labeling with ciliated and goblet cell markers. (Row 2, E-H) *Maackia amurensis* II labeling of differentiated NHBEs and distal trachea, lectin labeling alone and then lectin labeling with ciliated and goblet cell markers. The pseudo-colors are receptor lectins (green), goblet cells (magenta) and ciliated cells (red). Yellow indicates co-localization between a receptor lectin and the ciliated cell marker. White indicates co-localization between a receptor lectin and the goblet cell marker. Bar is 20 µm.
Figure 4 NY312-AF594 binding of NHBEs and distal trachea

Leica SP8 confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded cells and tissues. All imaging was done with a 40x oil objective at zoom 1.5, 2048 x 2048 pixels. (Row 1, A-C) NY312-AF594 bound to NHBE cells with apical cell markers. (Row 2, D-F) NY312-AF594 bound to human distal trachea with apical cell markers. The pseudo-colors are NY312-AF594 (green), goblet cells (magenta), ciliated cells (red), and nuclei (gray). Yellow indicates co-localization between NY312-AF594 and the ciliated cell marker. White indicates co-localization between NY312-AF594 and the goblet cell marker. Bar is 10 µm.
Figure 5 NY312 and CA04 NHBE infections

(A-D) Hematoxylin and eosin stained sections of differentiated NHBE cells infected with NY312, (A) 8 hours, (B) 24 hours, (C) 48 hours and (D) 72 hours post-infection respectively. Bar is 20 µm. (E) The replications kinetics for NY312 and CA04 NHBE infections were similar. Each point represents the average across three replicates for a virus at one time-point post-infection and bars are standard error.
Figure 6 Comparison of influenza antigen distribution by cell type Pandemic 2009 influenza infected NHBEs and human trachea

Leica SP8 laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded NHBE cells and human trachea. All imaging was done with a 40x oil objective at zoom 1.5, 2048 x 2048 pixels. The pseudo-colors are influenza antigen (green), goblet cells (magenta), ciliated cells (red) and basal cells (blue). Yellow indicates co-localization between influenza antigen and the ciliated cell marker. White indicates co-localization between influenza antigen and the goblet cell marker. Cyan indicates co-localization between influenza antigen and the basal cell marker. (A) CA04 infected NHBE cell layer at 24 hours post-infection. (B) Trachea from an autopsy of an individual who died from infection with a 2009 pandemic virus strain. (Row 2) Infected NHBE cells, (C) influenza antigen labeling only, followed by (D-F) influenza antigen merged with ciliated, goblet and basal cell markers individually. (Row 3) Infected human trachea, (G) influenza antigen labeling only, followed by (H-I) influenza antigen merged with ciliated, goblet and basal cell markers individually. Bar is 10 µm.
Figure S1 Jacalin co-localizes with Muc5Ac

Leica SP5 white light laser confocal maximum projection images of full thickness z-stack of immunofluorescence labeled formalin-fixed paraffin-embedded human distal tracheal tissue labeled with DAPI (blue), Jacalin lectin (green), and anti-Muc5Ac antibody (red) imaged with DIC image capture in sequence. All imaging was done with a 40x oil objective at zoom 1.0, 1024 x 1024 pixels. (A) DAPI and lumen is marked by a (*). (B) Jacalin labeled mucins in goblet cells with DAPI. (C) Muc5Ac labeled mucins in goblet cells with DAPI. (D) Merge of Jacalin, Muc5Ac and DAPI. The arrows denote yellow showing co-localization of the mucin markers. (E) DIC, single slice image, 0.42 μm thick, showing cell outlines. F: Merge showing that the mucin markers labeled goblet cells appropriately. White arrows denote apical epithelial aspect and black arrows point at the epithelial basal lamina. Bar is 20 μm.
Figure S2 NHBE cell types, example 1

Leica SP8 laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded differentiated NHBE cells. All imaging was done with a 40x oil objective at zoom 2.7, 2048 x 2048 pixels. (A) Ciliated cells (red) and nuclei (gray). (B) Goblet cells (magenta) and nuclei. (C) Basal cells (green) and nuclei. (D) Fluorescence merge. (E) DIC, representative single 0.20 µm thick slice showing cell outlines. (F) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar is 10 µm.
Figure S3 NHBE cell types, example 2

Leica SP8 laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded differentiated NHBE cells. All imaging was done with a 40x oil objective at zoom 2.7, 2048 x 2048 pixels. (A) Ciliated cells (red) and nuclei (gray). (B) Goblet cells (magenta) and nuclei. (C) Basal cells (green) and nuclei. (D) Fluorescence merge. (E) DIC, representative single 0.20 µm thick slice showing cell outlines. (F) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar is 10 µm.
Figure S4 Distal trachea cell types

Leica SP8 laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded distal trachea. All imaging was done with a 40x oil objective at zoom 2.7, 2048 x 2048 pixels. (Row 1 and 2) Images of individual markers (A) nuclei (gray), (B) ciliated cells (red), (C) goblet cells (magenta), (D) basal cells (green), (E) neuroendocrine cells (blue), and (F) macrophage/dendritic cell (cyan). (F) Fluorescence merge. (G) DIC, representative single 0.20 μm thick slice showing cell outlines. (H) Merge all showing cell type markers in relationship to DIC cell outlines (nuclear label removed). Bar is 10 μm.
Figure S5 Carina cell types

Leica SP8 laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded carina. All imaging was done with a 40x oil objective at zoom 2.7, 2048 x 2048 pixels. (Row 1 and 2) Images of individual markers (A) nuclei (gray), (B) ciliated cells (red), (C) goblet cells (magenta), (D) basal cells (green), (E) neuroendocrine cells (blue), and (F) macrophage/dendritic cell (cyan). (F): Fluorescence merge. (G) DIC, representative single 0.20 µm thick slice showing cell outlines. (H) Merge all showing cell type markers in relationship to DIC cell outlines (nuclear label removed). Bar is 10 µm.
Figure S6 Average epithelial heights of distal trachea, carina and NHBE cells

Box plots of epithelial height measurements for distal trachea, carina and NHBE cells, n=30 for each. A one way ANOVA with means comparison by Tukey test demonstrated that all means were significantly different from each other at the 0.05 level.
Figure S7 Neuraminidase treatment removes sialic acids from NHBEs and distal trachea

Leica SP8 laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded NHBEs and distal trachea. All imaging was done with a 40x oil objective at zoom 2.0, 2048 x 2048 pixels. Slides were labeled with either biotinylated-*Sambucus nigra* (SNA) or *Maackia amurensis* II (MAA II) lectins. SNA binding to NHBEs (A-B), (B) was pre-treated with 8 UN/ml neuraminidase (NA) for 90+ minutes prior to lectin application, and distal trachea (C-D), (D) was pre-treated with 4 UN/ml NA for same time as prior. MAA II binding to NHBEs (E-F), (E) was pre-treated with 8 UN/ml neuraminidase (NA) for 90+ minutes prior to lectin application, and distal trachea (G-H), (H) was pre-treated with 4 UN/ml NA for same time as prior. Bar 30 µm.
Figure S8 *Sambucus nigra* on NHBEs

Leica SP5 white light laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded cells. All imaging was done with a 40x oil objective at zoom 2.0, 2048 x 2048 pixels. (A) SNA (green). (B) Goblet cells (magenta). (C) Ciliated cells (red). (D) Fluorescence merge with addition of nuclei (gray). (E) DIC, representative single 0.21 μm thick slice showing cell outlines. (F) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar 20 μm.
Figure S9 *Sambucus nigra* on distal trachea sample 1

Leica SP5 white light laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded distal trachea tissue. All imaging was done with a 40x oil objective at zoom 2.0, 2048 x 2048 pixels. (A) SNA (green). (B) Goblet cells (magenta). (C) Ciliated cells (red). (D) Fluorescence merge with addition of nuclei (gray). (E) DIC, representative single 0.21 μm thick slice showing cell outlines. (F) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar 20 μm.
Figure S10 *Sambucus nigra* on distal trachea sample 2

Leica SP5 white light laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded distal trachea tissue. All imaging was done with a 40x oil objective at zoom 2.0, 2048 x 2048 pixels. (A) SNA (green). (B) Goblet cells (magenta). (C) Ciliated cells (red). (D) Fluorescence merge with addition of nuclei (gray). (E) DIC, representative single 0.21 μm thick slice showing cell outlines. (F) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar 20 μm.
Figure S11 *Sambucus nigra* on carina matched with distal trachea sample 2

Leica SP5 white light laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded carina tissue. All imaging was done with a 40x oil objective at zoom 2.0, 2048 x 2048 pixels. (A) SNA (green). (B) Goblet cells (magenta). (C) Ciliated cells (red). (D) Fluorescence merge with addition of nuclei (gray). (E) DIC, representative single 0.21 μm thick slice showing cell outlines. (F) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar 20 μm.
Figure S12 *Maackia amurensis* II on NHBES

Leica SP5 white light laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded cells. All imaging was done with a 40x oil objective at zoom 2.0, 2048 x 2048 pixels. (A) MAA II (green). (B) Goblet cells (magenta). (C) Ciliated cells (red). (D) Fluorescence merge with addition of nuclei (gray). (E) DIC, representative single 0.21 µm thick slice showing cell outlines. (F) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar 20 µm.
Figure S13 *Maackia amurensis* II on distal trachea sample 1

Leica SP5 white light laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded distal trachea tissue. All imaging was done with a 40x oil objective at zoom 2.0, 2048 x 2048 pixels. (A) MAA II (green). (B) Goblet cells (magenta). (C) Ciliated cells (red). (D) Fluorescence merge with addition of nuclei (gray). (E) DIC, representative single 0.21 µm thick slice showing cell outlines. (F) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar 20 µm.
Figure S14 *Maackia amurensis* II distal trachea sample 2

Leica SP5 white light laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded distal trachea tissue. All imaging was done with a 40x oil objective at zoom 2.0, 2048 x 2048 pixels. (A) MAA II (green). (B) Goblet cells (magenta). (C) Ciliated cells (red). (D) Fluorescence merge with addition of nuclei (gray). (E) DIC, representative single 0.21 μm thick slice showing cell outlines. (F) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar 20 μm.
Figure S15 *Maackia amurensis* II on carina matched with distal trachea sample 2

Leica SP5 white light laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded carina tissue. All imaging was done with a 40x oil objective at zoom 2.0, 2048 x 2048 pixels. (A) MAA II (green). (B) Goblet cells (magenta). (C) Ciliated cells (red). (D) Fluorescence merge with addition of nuclei (gray). (E) DIC, representative single 0.21 μm thick slice showing cell outlines. (F) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar 20 μm.
Figure S16 *Maackia amurensis* I on NHBE cells and distal human trachea

Leica SP5 white light laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded carina tissue. All imaging was done with a 40x oil objective at zoom 2.0, 2048 x 2048 pixels. (Row 1) NHBE cells (A) MAA I only (green) labeling only and (B) MAA I with apical cell type markers for goblet cells (magenta) and ciliated cells (red) NHBE cells; (C-D) the same with addition of neuraminidase pre-treatment. (E-H) shows the same immunofluorescence on distal trachea. Yellow indicates co-localization between a receptor lectin and the ciliated cell marker. White indicates co-localization between a receptor lectin and the goblet cell marker. Bar 20 µm.
Figure S17 NY312-AF594 binding to NHBE cells

Leica SP8 confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded cells. All imaging was done with a 40x oil objective at zoom 1.5, 2048 x 2048 pixels. Row 1: Single immunofluorescence images (A) NY312-AF594 (green), (B) goblet cell marker (magenta), and (C) ciliated cell marker (red). D: Fluorescence merge with addition of nuclei (gray). E: DIC, representative single 0.21 \( \mu \text{m} \) thick slice showing cell outlines. F: Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar is 10 \( \mu \text{m} \).
Figure S18 NY312-AF594 binding to distal trachea

Leica SP8 confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded human distal trachea. All imaging was done with a 40x oil objective at zoom 1.5, 2048 x 2048 pixels. (Row 1) Single fluorescence images: (A) NY312-AF594 (green), (B) goblet cell marker (magenta), and (C) ciliated cell marker (red). (D) Fluorescence merge with addition of nuclei (gray). (E) DIC, representative single 0.21 µm thick slice showing cell outlines. (F) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar is 10 µm.
Figure S19 NY312-AF594 and SNA compete for the same receptors

Leica SP8 laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded human trachea. All imaging was done with a 40x oil objective at zoom 0.75, 2048 x 2048 pixels. (Row 1) Virus applied before SNA: (A) NY312-AF594, (B) SNA, and (C) merge with nuclei (blue). (Row 2) SNA applied before virus: (D) NY312-AF594, (E) SNA, and (F) merge with nuclei. Note that virus labeling in (Row 1) is stronger than in (Row 2) and SNA labeling is stronger in (Row 2) than in (Row 1). Bar is 20 µm.
Figure S20 Influenza antigen distribution by cell type in CA04 Infected NHBEs

Leica SP8 laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded NHBE cells. All imaging was done with a 40x oil objective at zoom 1.5, 2048 x 2048 pixels. (Row 1) Single fluorescence images: (A) NY312-AF594 (green), (B) goblet cell marker (magenta), (C) ciliated cell marker (red) and (D) basal cell marker (blue). (E) Fluorescence merge. (F) Same merge with addition of nuclei (gray). (G) DIC, representative single 0.21 mm thick slice showing cell outlines. (H) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar is 10 µm.
Figure S21 Non-specific background signal with anti-influenza antibody labeling

Leica SP8 laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded NHBE cells and human trachea. All imaging was done with a 40x oil objective at zoom 2.0, 2048 x 2048 pixels. The pseudo-colors are influenza antigen (green) and nuclei (blue).

Row 1: NHBE cells, (A) infected with CA04, 24-hour post-infection and (B) uninfected NHBE cells. Row 2: Human tracheal tissue, (C) infected with pandemic 2009 strain virus and (D) uninfected human trachea control tissue. Bar is 20 µm.
Figure S22 Influenza antigen distribution by cell type in Pandemic 2009 influenza infected human trachea

Leica SP8 laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin-embedded human trachea. All imaging was done with a 40x oil objective at zoom 1.5, 2048 x 2048 pixels. (Row 1) Single fluorescence images: (A) NY312-AF594 (green), (B) goblet cell marker (magenta), (C) ciliated cell marker (red) and (D) basal cell marker (blue). (E) Fluorescence merge. (F) Same merge with addition of nuclei (gray). (G) DIC, representative single 0.21 mm thick slice showing cell outlines. (H) Merge all showing cell type markers in relationship to DIC cell outlines (nuclei label removed). Bar is 10 µm.
References


Chan RW, Chan MC, Nicholls JM & Malik Peiris JS (2013) Use of ex vivo and in vitro cultures of the human respiratory tract to study the tropism and host responses of highly pathogenic avian influenza A (H5N1) and other influenza viruses. Virus Res 178:133-145

Chan RW, Yuen KM, Yu WC, Ho CC, Nicholls JM, Peiris JS & Chan MC (2010) Influenza h5n1 and h1n1 virus replication and innate immune responses in bronchial epithelial cells are influenced by the state of differentiation. PLoS One 5:e8713


Hovenberg HW, Davies JR & Carlstedt I (1996) Different mucins are produced by the surface epithelium and the submucosa in human trachea: Identification of muc5ac as a major mucin from the goblet cells. Biochem J 318 ( Pt 1):319-324


Ilyushina NA, Bovin NV & Webster RG (2012a) Decreased neuraminidase activity is important for the adaptation of H5N1 influenza virus to human airway epithelium. J Virol 86:4724-4733


(2005) The viennese culture method: Cultured human epithelium obtained on a
dermal matrix based on fibroblast containing fibrin glue gels. Burns 31:25-29

Kesimer M, Kirkham S, Pickles RJ, Henderson AG, Alexis NE, Demaria G, Knight D,
Thornton DJ & Sheehan JK (2009) Tracheobronchial air-liquid interface cell culture:
A model for innate mucosal defense of the upper airways? Am J Physiol Lung Cell
Mol Physiol 296:L92-L100

Kogure T, Suzuki T, Takahashi T, Miyamoto D, Hidari KI, Guo CT, Ito T, Kawaoka Y &
Suzuki Y (2006) Human trachea primary epithelial cells express both sialyl(alpha2-3)gal receptor for human parainfluenza virus type 1 and avian influenza viruses, and

improved lectin-based method for the detection of mucin-type o-glycans in biological
samples. Analyst 138:3522-3529

transmembrane conductance regulator trafficking modulates the barrier function of
airway epithelial cell monolayers. J Physiol 588:1195-1209

Lopez-Souza N, Favoreto S, Wong H, Ward T, Yagi S, Schnurr D, Finkbeiner WE,
susceptibility to rhinovirus infection is greater for bronchial than for nasal airway

Martinez-Anton A, Sokolowska M, Kern S, Davis AS, Alsaaty S, Taubenberger JK, Sun J,
microrna and mRNA expression with differentiation of human bronchial epithelial

Matrosovich MN, Matrosovich TY, Gray T, Roberts NA & Klenk HD (2004) Human and
avian influenza viruses target different cell types in cultures of human airway
epithelium. Proc Natl Acad Sci U S A 101:4620-4624

Menscher A 2013. Chapter 17. The respiratory system. Junqueira's basic histology: Text and


spread through human airway epithelium at temperatures of the proximal airways. PLoS Pathog 5:e1000424


123

Wong AP, Keating A & Waddell TK (2009) Airway regeneration: The role of the clara cell secretory protein and the cells that express it. Cytotherapy 11:676-687


CHAPTER 4

Binding and entry of 1918 Pandemic Influenza Receptor Binding Domain Variants in Normal Human Bronchial Epithelial Cells
Abstract

The 1918 “Spanish” influenza pandemic resulted in approximately 50 million deaths and is the most severe influenza pandemic on record to date. Many questions remain regarding its origin, mechanisms of adaptation, and enhanced pathogenicity in humans. We used the human trachea derived primary cell line, normal human bronchial epithelial (NHBE) cells, to examine the binding and entry pattern of 5 1918 receptor binding domain variants: A/1/SC/1918 (SC), A/1/NY/1918 (NY), A/1/1918/VA (VA), A/1/1918/NY3 (NY3), and the ‘avianized’ 1918 virus. Prevailing hypotheses postulate that the hemagglutinin protein of avian-adapted influenza viruses binds via α2,3 sialic acid (Sia)-linked glycans and that the mammalian-adapted viruses bind α2,6 Sia-linked glycans, and that host switch requires a complete change in this Sia preference. SC exclusively binds and VA is predicted to bind α2,6, NY binds mixed α2,3/α2,6, the binding preference of NY3 is unknown and the ‘avianized’ virus exclusively binds α2,3 Sia. We hypothesized that these viruses’ binding and entry profiles would align with their Sia preferences as well as show a distinct cell type tropism correlating with the Sia receptor distribution on the NHBE cells. Contrary to our expectations, all 5 viruses bound and entered the cells and AV was the most effective, easily entering both goblet and ciliated cells, including cells that express only α2,6 Sia on their surface. AV also outperformed NY and SC in an NHBE multi-cycle replication kinetics study at the 24 and 36 hr time-point, yet did not have a stronger hemagglutinin binding capability as measured by hemagglutinin assay.
Introduction

Influenza A viruses cause acute respiratory viral disease in both annual (seasonal) epidemics and infrequent pandemics (Wright PF, 2007). The 1918 “Spanish” influenza pandemic resulted in approximately 50 million deaths globally and is the most severe influenza pandemic on record to date (Johnson and Mueller, 2002; Taubenberger and Morens, 2006). Many questions remain regarding its origin, mechanisms of adaptation, and enhanced pathogenicity in humans (Taubenberger and Kash, 2011). 1918 pandemic influenza (1918) pathogenesis research has relied on the development of appropriate \textit{in vitro} and \textit{in vivo} experimental animal studies.

Recently, differentiated normal human bronchial epithelial (NHBE) cells have been used for influenza A research (Thompson et al., 2006) and (Chapter 3). Their validation as a model for distal trachea revealed some limitations in their application, but confirmed that they are a good model for studying acute influenza A infections within human distal trachea (Chapter 3).

Influenza A viruses bind to terminal sialic acids on target cell glycans. It is hypothesized that changes in the influenza A virus hemagglutinin protein (HA) receptor binding domain (RBD) are important in the process of host adaptation, specifically allowing avian-origin influenza A viruses to adapt to humans. The 1918 HA gene has been sequenced from a number of different postmortem samples, and several 1918 HA RBD variants have been reported (Glaser et al., 2005; Reid et al., 1999; Sheng et al., 2011; Tumpey et al., 2007). In addition to
A/South Carolina/1/1918 (SC), which has an aspartic acid at both positions 187 and 222 in HA1 conferring an α2,6 sialic acid receptor-binding specificity, and A/NY/1/1918 (NY), which differs from SC by encoding a glycine at position 222 conferring a mixed α2,3/α2,6 binding specificity, Sheng et al., 2011 reported two new variants with yet to be confirmed RBD specificities (Table 1). Based on the computational modeling results the HA RBD of A/VA/1/1918 (VA), which in addition to aspartic acids at positions 187 and 222 has a change from glutamine to arginine at position 189 in the HA1 domain, may have an enhanced α2,6 binding specificity (Sheng et al., 2011; Stevens et al., 2006). No information is available for the A/NY/3/1918 (NY3) wherein deep sequencing revealed a predominance of asparagine rather than glycine at position 222. Finally, the ‘avianized’ version of the 1918 virus (AV) in which the aspartic acid at position 187 in NY was mutagenized back to the avian influenza virus consensus glycine, was reported to be exclusively α2,3 (Glaser et al., 2005). How this sialic acid binding preference correlates with the cell type entered and infected within the distal trachea is poorly understood. We know from autopsy reviews that sialic acid distribution as determined by lectin histochemistry is a poor predictor of what cell types are infected at time of death attributed to influenza A infection (Gill et al., 2010; Sheng et al., 2011). In fact, by then all respiratory epithelial cell types except the basal cells in damaged bronchial and tracheal epithelia tend to be involved. Based on lectin histochemistry, the human distal trachea was reported to display predominantly α2,6 sialic acids on its apical epithelial surface (Nicholls et al., 2008; Shinya et al., 2006) and (Chapter 3). Similarly determined, NHBEs were variably reported to display exclusively α2,6 sialic acids or a
mixture of $\alpha_{2,3}/\alpha_{2,6}$ sialic acids (Chan et al., 2013; Chan et al., 2010; Ibricevic et al., 2006; Kogure et al., 2006; Matrosovich et al., 2004) and (Chapter 3). However, in addition to type of terminal sialic acid attached to galactose, glycan topology appears to play a role (Chandrasekaran et al., 2008). Recent glycomic analysis of human lung by mass spectrometry confirmed the presence of a wide variety of both N- and O-linked glycans, both $\alpha_{2,3}$ and $\alpha_{2,6}$, that interestingly correlated poorly with those found on the glycan arrays currently employed to determine viral binding preferences (Walther et al., 2013). Given so many unknowns, we narrowed our focus in this study to the examination of the binding and entry patterns of the 5 aforementioned RBD variants of 1918 at the cell type level in the NHBE model. We asked whether at the time of binding and early entry, 1918 influenza viruses with RBD polymorphisms differ in their affinity for human airway epithelial cells. Additionally, do certain variants preferentially bind and enter specific cell types?

To investigate these questions, we infected differentiated NHBEs with the 5 1918 RBD variants at a constant multiplicity of infection (MOI) and constant hemagglutinin assay unit. We then examined these infections at a variety of time points, including 5 and 20 minutes post application of virus and in an infection time-slowed model, wherein virus was applied to the cells while they were chilled on ice. Our analysis was based on a multi-label immunofluorescence model for influenza antigen distribution at the cell type level in formalin fixed paraffin embedded infected NHBE cells (Chapter 3) as well as studies that considered determination of the location of virus within the endocytic pathway during early
stages of infection. Finally, we validated that the NHBEs were capable of sustaining multi-cycle replication for the viruses under study.
Materials and Methods

Growth and differentiation of cells

Primary Normal Human Bronchial/Tracheal Epithelial (NHBE) cells (CC-2541, Lonza; Walkersville, MD) from a single donor were grown as per manufacturer’s instructions as described in detail in the prior chapter.

Construction and rescue of chimeric viruses

Five variants of 1918 influenza A hemagglutinin receptor binding domain (RBD) virus were generated, four previously reported and the fully avianized version (Glaser et al., 2005; Reid et al., 1999; Sheng et al., 2011). Table 1 shows each HA RBD variant’s amino acid sequence and its hemagglutinin sialic acid binding preference (if known). Furthermore, all viruses are referred to as SC, NY, NY3, VA, and AV respectively (Table 1). In summary, all viruses but AV have aspartic acid (D) at position 187, AV has glutamic acid (E) at this position. At position 222, SC and VA have D, NY and AV have glycine (G), and NY3 has asparagine (N) dominant as determine by deep sequencing (Sheng et al., 2011) but D as well. VA is the same as SC but additionally has an arginine (R) at position 189 instead of the consensus glutamine (Q). The fully reconstructed 1918 H1N1 influenza virus was prepared by reverse genetics as previously described (Qi et al., 2009). RBD mutations in the 1918 HA gene for each of the other viruses were generated with a site-directed mutagenesis kit following the manufacturer's instructions (Stratagene, La Jolla, CA). All rescued viruses were propagated once in Madin-Darby canine kidney (MDCK) cells (ATCC; Manassas, VA). The genomic sequence of each rescued virus was then confirmed by sequence analysis of the inoculum.
All viruses and infectious samples were handled in accordance with the select agent guidelines of the National Institutes of Health and the Centers for Disease Control and Prevention under the supervision of the NIH Select Agent and Biosurety Programs and the NIH Department of Health and Safety.

Hemagglutinin Assay

We normalized our viral infections using multiplicity of infection (MOI). However, considering our experimental focus was binding and entry we decided it was also prudent to consider normalization of the viruses by hemagglutinin assay unit. Therefore a standard hemagglutinin assay was conducted on the AV, SC, and NY viruses (Hirst, 1942).

Viral infections

Uninfected control inserts were immersed in 10% neutral buffered formalin (NBF) prior to transporting the rest of the cells into the select agent suite where infections were conducted. In order to examine variation in binding and entry patterns of the five viruses, infections were initially conducted as a single experiment with 3.0 and 1.0 multiplicities of infection (MOI) for all five viruses in triplicate (three inserts per MOI/virus combination). Mucus was aspirated from each well, cells were washed with 300 ul PBS three times, 100 ul of each virus at the appropriate MOI dil in PBS was applied, cells were incubated for 20 min at 37°C 5% CO₂, virus was aspirated, cells were washed once with PBS, and inserts placed in NBF. After 48 hours, fixed inserts were removed from the select agent suite.
When we discovered that these infections resulted in binding and entry of virus into both ciliated and goblet NHBE cells across all RBD variant viruses with no clear pattern of variation, we conducted a second experiment with all 5 viruses in the same manner as above where we both reduced the MOI and the incubation time, 0.1 and 0.01 for a 20 min incubation period and 1.0, 0.1, and 0.01 for a 5 min period.

Replication kinetics
Replication kinetics including the 12, 24 and 36-hour time-points were determined for AV, SC, and NY viruses. Cells were infected with 300 ul of viral solution at MOI 0.1 and cells incubated for the appropriate number of hours. This experiment was conducted in triplicate for each time-point/virus combination. At the appropriate times for each infected insert, apical media were collected by adding 200 ul PBS to each biological replicate then collecting all fluid at the apical aspect, placing the insert in NBF and collecting the basal media (fluid remaining in the well). All supernatants were stored individually in cryovials at -80°C. Standard plaque assays for influenza A (Gaush and Smith, 1968) were conducted on these fluids.

Real-time quantitative PCR on cell lysates
Additionally, we determined viral load (intracytoplasmic and plasma membrane bound) by quantitative real time-PCR for the influenza A matrix protein 1 (M1) gene using cell lysates from a parallel experiment with all 5 viruses at MOI 0.01 and MOI 0.1 and both the 5 min and 20 min incubation time periods, conducted in triplicate as described above. Briefly, total
RNA was isolated from individual infected NHBE samples and their viral loads estimated as previously described (Kash et al., 2011). Results were graphed as a threshold cycle ratio of the calibrator glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) over M1. Two way ANOVAs with Tukey’s Multiple Comparison Tests was used for statistical analysis of both the replication kinetics and cell lysate real-time PCR data with alpha set to 0.05 and 0.01 respectively. Graphs and analysis were done in Prism 6.0c (GraphPad; La Jolla, CA).

Immunofluorescence
The fixed, differentiated infected and uninfected cells on their membranes were individually embedded on edge in paraffin. Approximately 5 µm thick cross sections of differentiated cells were cut and placed on positively charged slides, two sections per slide (NC State University College of Veterinary Medicine Histopathology Lab; Raleigh, NC). Each slide included two sections from a given well.

In order to determine which epithelial cell types were bound and entered by the influenza A viruses, sections for each MOI/virus combination were labeled following the immunofluorescence for influenza antigen with cell type markers on NHBE cells protocol described in Chapter 3 with the following changes. The donkey serum block was reduced to 5% strength because the lot of the anti-influenza antibody lacked the background issues documented in Chapter 3. Goblet cells were detected with 2 µg/ml Biotinylated Jacalin (B-1155, Vector Labs; Burlingame, CA) and visualized with 10 µg/ml of Streptavidin Alexa Fluor-488 conjugate (S-11223, Molecular Probes; Eugene, OR), instead of fluorochrome
conjugated Jacalin. Ciliated and basal cells were detected as before but visualized with Dylight-549 AffiniPure Donkey Anti-Mouse (discontinued; Jackson Immunoresearch (JI); West Grove, PA) and Dylight-649 AffiniPure Donkey Anti-Rabbit (discontinued, JI) respectively.

To determine the cellular compartment in which the influenza antigen signal was located, we used immunofluorescence for endocytic pathway markers. Slides were prepped for antibody application as described in the previously (Chapter 3). We made dual labels with influenza A antibody and the early endosome marker, rabbit polyclonal Anti-Rab5 antibody (ab13253, Abcam; Cambridge, MA), or the lysosome marker, mouse monoclonal antibody Anti-LAMP1 antibody [H4A3] (ab25630, Abcam), as well as a triple label with both endosome markers and influenza A antibody. Rab5 was applied at 1:50 dil and Lamp1 at 2 µg/ml both dil in TBS. Appropriate secondary pairings for each of the markers were selected in accordance with the previously described immunofluorescence work (Chapter 3). All slides were mounted in Prolong Gold (P36930, Life Technologies; Grand Island, NY) as per vendor instructions then visualized and images captured on a Leica TCS SP5 X White Light Laser confocal system (Leica Microsystems; Buffalo Grove, IL) configured with Argon laser, 405 nm diode and photomultiplier tube detectors.

Image capture and analysis

Individual confocal sessions focused on an entire batch, defined as a single MOI/incubation time. In this manner, image capture settings were as constant as possible intra-batch. Also,
settings were reused between sessions, re-checking the thresholding of the influenza antigen signal against batch specific uninfected control slides at each session start. Slides from the first experiment, MOI 3.0 and 1.0 with 20 min incubation, were reviewed manually with representative images taken for each virus/MOI.

For the second experiment, each well for each virus/MOI combination was reviewed manually, visually scanning both strips of cells on the slide. Minimally three representative 63x oil objective at zoom 1, 1024 x 1024 pixels z-stacks were taken. Additionally, a 63x oil objective at zoom 2.5, 1024 x 1024 pixels representative z-stack was captured for each virus/MOI combination was taken. When we discovered that there was little difference between MOI 0.1 and 0.01 we focused our analysis on 0.01. We also decided to focus on the 5 min time point due to great similarity between it and the 20 min time point.

Image post processing and analysis was done with Leica Application Suite (Leica Microsystems) and Imaris 7.6.3 (Bitplane; Zurich, Switzerland). Max projected fluorescent images for influenza antigen with and without cell type markers were created. Within an experimental batch, intensity levels were adjusted consistently across all samples. Additionally, the differential interference contrast (DIC) slice wherein ciliated and goblet cells were near equivalently in focus for the largest number of cells was selected from each z-stack and merged with a max projected image of multi-channel fluorescence. These images were reviewed for presence of influenza A antigen by cell type. Final figure compilation was done in Adobe Photoshop CS6 Extended (Adobe, San Jose, CA).
Results

The ‘avianized’ 1918 virus bound and entered NHBE cells most efficiently. All infections at all MOI and incubation time combinations resulted in binding and entry of virus in all samples. Virus infections at MOI 3.0 or 1.0 incubated for 20 minutes resulted in intracytoplasmic influenza antigen from nearly every apical epithelial cell. This prompted our reduction in both MOI and incubation time. We began to appreciate variance in the virus entry patterns at MOI 0.1, even more so at MOI 0.01, especially for the 5 min incubation time. Therefore infection at MOI 0.01 for 5 min was the main focus of our detailed analysis along with cross checks with the 20 min material from the same MOI. Representative images for each virus at MOI 0.01 at the 5 min time-point are shown in Figure 1.

Influenza antigen was intracytoplasmic as well as bound to the apical epithelial surface of both goblet and ciliated cells. The intracytoplasmic influenza antigen signal was often more prominent in the goblet cells. The relative intensity of influenza antigen labeling across the entire epithelium was highest for AV, lowest for SC, and intermediary for the other three viruses. For AV, all cells identified as goblet and ciliated cells except a single ciliated cell were positive for influenza antigen intracytoplasmically. In general, virus was less apparent intracytoplasmically in ciliated cells than goblet cells. Virus was also bound to the external aspect of the plasma membrane more prominently at the base of cilia than the surface of goblet cells. Overall, intracytoplasmic influenza antigen was more prominent in goblet cells for SC. However, it also entered ciliated cells and was bound at the base of the cilia. NY, was quite similar to AV in that frequently the cell lacking an intracytoplasmic influenza antigen signal was of the ciliated cell type and influenza antigen was not always visibly
bound to the apical aspect of the cell. Confirmed goblet cells were all positive for intracytoplasmic influenza antigen for NY. NY3 had nearly as many cells with an intracytoplasmic influenza antigen signal as AV. There was a single goblet cell and two ciliated cells that were negative, with bound influenza antigen at the apical aspect of one of the ciliated cells. VA was similar to NY3 but had several more intracytoplasmically negative ciliated cells, some of which also lacked bound influenza. These observations remain descriptive only due to the sample size and because four of the five viruses varied little in terms of uninfected cells. When the intracytoplasmic location of the influenza antigen was explored using endosomal markers, there was occasional co-localization with early (Rab5) and more co-localization with late (Lamp1) endosomal markers as well as a noticeable portion of intracytoplasmic influenza antigen signal that failed to co-localize with either marker (Fig. 2).

Three viruses’ hemagglutinin binding capabilities were nearly equivalent. We selected AV, NY and SC, the viruses to represent the range of range of influenza antigen levels on immunofluorescence and conducted hemagglutination assays in order to examine if the reason for this divergence was due to variance in the HA binding capability of these viruses. Once stock virus titer is taken into consideration, the HA unit values are nearly equivalent (Table 2).
Three viruses were capable of multi-cycle growth

Multi-cycle growth kinetics assays demonstrated that the AV, SC, and NY viruses were replication competent (Fig. 3). There was no statistical difference between viral titers at 12 hours, but at 24 and 36 hours AV replicated to significantly higher titers as compared to the NY and SC.

Real-time quantitative PCR confirmed that the ‘avianized’ virus is more abundant in cells. Influenza viral RNA was present bound to and inside cells at both the 5 and 20 min time-points for all viruses at both an MOI of 0.1 and 0.01 (Fig. 4, 5). The relative amounts were the same across all viruses, MOIs and incubation times except that AV viral RNA was present in statistically significantly higher amounts than each of the other four viruses at an MOI 0.01 at 5 min (Fig. 5). By 20 min, this difference was no longer statistically significant.

In summary, all five viruses bound and entered both apical epithelial cell types. AV, an $\alpha_{2,3}$ binding preference only virus, readily entered cells despite the cells expressing primarily $\alpha_{2,6}$ on their surfaces. AV was also the most abundant intracellularly and replicated more efficiently than NY and SC.
Discussion

All five 1918 receptor binding domain (RBD) variants bound and entered the fully differentiated normal human bronchial epithelial (NHBE) cells within 5 minutes. We have shown this visually by influenza antigen immunofluorescence (IF) (Fig. 1, Column 1) and confirmed these observations by real-time qPCR on cell lysates (Figure 4, 5). Even at an MOI of 0.01 (the lowest used), many luminal cells (ciliated and goblet cells) were positively labeled for influenza antigen intracytoplasmically as well as on their apical epithelial surfaces. Curiously, the virus that most efficiently entered the NHBE cells, as measured by IF and qPCR was the ‘avianized’ virus (AV). Additionally, A/VA/1/1918 (VA) predicted by molecular modeling to have an α2,6 sialic acid (Sia) binding preference and therefore hypothesized to behave like A/1/1918/SC (SC), which is confirmed to have this preference, outperformed SC based on the IF data.

The swiftness of viral entry for all 5 viruses in our experiments with the 20 min incubation time, while biologically explainable based on the life-cycle of influenza prevented us seeing any differences between the viruses (Wright et al., 2007). Consequently, we conducted an additional series of experiments with a 5 min incubation time in order to test for evidence of any differentiation in entry ability. Additionally, when for many viruses, the majority of the luminal cells were positive for intracytoplasmic influenza antigen in our infection studies at the MOIs of 3.0 and 1.0, we decreased our MOI to 0.1 and 0.01. These later experiments confirmed the patterns we had begun to see at MOI 1.0, and provided further separation between the viruses’ binding and entry patterns than we initially appreciated. However, we
recommend that any future experiments with these viruses that are focused on binding only, should be conducted on ice, because while virus is visibly bound at 5 min, the bound virus may not be representative of the initial binding pattern.

By qPCR, at the 5 min time-point for an infection at an MOI of 0.01, the virus that bound and entered cells most efficiently was AV (Fig. 5). By immunofluorescence, the relative intensity of the intracytoplasmic influenza antigen signal for AV was also the strongest, that for NY, VA and NY3 intermediary, and SC clearly the weakest (Fig.1). Figure 1 shows representative images, maximum projections of a z-stacks through the full thickness of each sample, of single wells from the 5 min incubation time, MOI 0.01 infections. These images while only representative of the relative influenza antigen intensity across samples sectioned from each of the three individually infected wells for each viruses were carefully selected. Where there was inter- or intra-well variance in the influenza antigen intensity, a field representative of the median relative intensity was selected.

We also determined that the AV advantage was not attributable to a stronger hemagglutinin binding capability as determined by hemagglutinin assay. SC and NY actually had stronger hemagglutination binding results than AV, even when the lower titer for AV stock virus used was compared with that of NY and SC (Table 2). Additionally, we confirmed the sequences of the 5 viruses in order to eliminate the possibility of human error or change in an RBD sequence during cell passage. All sequences confirmed as expected. Overall, these
confirmatory experiments along with corroborating IF data for other experimental incubation times and MOIs (not shown) convinced us that the AV entry phenomenon was legitimate. In contrast, we originally hypothesized that SC, with its confirmed α2,6 Sia binding preference would bind and enter the differentiated NHBE cells most readily. Not so (Fig. 1). Additionally, while entering more effectively than SC, the other virus with a similar predicted Sia preference, VA, was intermediary in its entry capability. However, VA’s Sia binding preference is unconfirmed by glycan array hybridization, so this virus may still be shown to have affinity for some α2,3 glycans. Our NHBE cells display more α2,6 Sia on both their ciliated and goblet cells than α2,3 Sia, similarly to human distal trachea and carina, the cell’s source tissue (Chapter 3).

Perhaps, the slower entry of SC into ciliated cells can be attributed to the presence of α2,3 Sia on some ciliated cells, as demonstrated by receptor lectin labeling in Chapter 3. However, we must also then consider that the same cells labeled for α2,6 Sia as well, since ciliated cells universally labeled for this (Chapter 3). Also, when you contrast the SC findings with the α2,3 Sia preferring AV’s findings, we are left with no way to explain our observations based on the Sia distribution on the NHBE epithelial surface. Interestingly, the difference seen with IF for SC was not apparent in the cell lysate viral RNA results. SC was not significantly different from VA, NY or NY3 (Fig. 4,5). Perhaps more virus was bound and not yet entered in the SC infected samples in comparison to those of the other three
viruses. If so, some of this bound virus may have been lost during embedding and processing of our samples for microscopy, but still measured in the real-time qPCR assay on cell lysates. This is not the first time an α2,3 Sia binding preference virus has been reported infecting NHBE cells. Oshansky et al., 2011 showed that low pathogenic avian influenza viruses with α2,3 binding preference can infect differentiated NHBE cells displaying only α2,6 Sia (Oshansky et al., 2011). Also when NHBE cells have been treated with neuraminidase to remove the Sia, both avian and human influenza viruses still readily infect them and there are similar findings for an H5N1 virus in ex vivo human respiratory tissues (Nicholls et al., 2007;Oshansky et al., 2011;Thompson et al., 2006). Additionally, this is not an in vitro restricted finding. Mice lacking the enzyme ST6Gal I sialyltransferase and therefore unable to attach α2,6 sialic acid to their cell surface N-linked glycoproteins and wild type mice, infected with human influenza have similar viral loads in their lungs (Glaser et al., 2007). This evidence as well as our findings suggest that we should look for other as of yet unidentified influenza receptors and co-receptors.

Many other viruses use a 2-step infection process. Examples include Reoviruses, which use glycans and junctional adhesion molecule (Danthi et al., 2013). Virus binds an abundant (primary) receptor, via a low affinity interaction (e.g. Sia), that brings the virus into contact with a less abundant receptor or co-receptor required for entry. Such a system for influenza has been proposed based on evidence over the last decade (Sun and Whittaker, 2013). Possibilities include a receptor involved in virus internalization after its initial binding to sialic acid or the presence of the sialic acid on a yet to be identified critical protein, also
involved in viral entry. Perhaps our focus should shift to identifying these purported co-receptors.

Another interpretation focuses on mucus. Prior to infection, the NHBE cells were thoroughly washed in order to remove mucus. During the growth and differentiation process, once they were placed in the air-liquid interface and began to differentiate into ciliated and goblet cells, mucus removal via PBS washes was necessary every 48 hr removed to maintain healthy cells (Chapter 3). This mucus is known to be rich in O-linked α2,3 sialic acids that has been shown to bind influenza viruses with an α2,3 Sia binding preference (Baum and Paulson, 1990). Perhaps, AV’s enhanced entry into our NHBE cells is not due to an epithelial binding advantage but rather the absence of mucus.

When our findings are considered within the wider context of evidence that human viruses have predominantly α2,6 Sia affinity or minimally mixed α2,3 /α2,6 while avian viruses prefer α2,3 Sia receptors, perhaps the greatest advantage isn’t even conveyed during initial host defense evasion, binding and entry. Influenza’s hemagglutinin protein is itself glycosylated. Glycosylation of the globular head (binding portion) can vary markedly by number, location and type of oligosaccharide (Baum and Paulson, 1990). The current NHBE model doesn’t account for carbohydrate interactions with the wider immune system. Perhaps, we should consider the possibilities of an advantage conveyed during viral release through co-adaptation of the viral hemagglutinin and neuraminidase genes (Baum and
Paulson, 1991; Kobasa et al., 1999) or even transmission through interaction of glycans within aerosolized droplets.

We used a low MOI in order to differentiate between our viruses’ binding and entry patterns. Viral titer represents only replication competent virions. Therefore, at an MOI of 0.01 there was approximately only 1 infective virion per 100 luminal cells. However, many more than 1 in 100 cells had a positive influenza antigen signal at 5 min. Although there is no specific published data and a technique for getting at the answer is not available to the authors’ knowledge, like many other influenza researchers we believe that the percentage of replication incompetent viruses likely greatly outstrips the percentage that are replication competent and our data supports this. Presumably, there are virions capable of binding and entry, but not necessarily the latter stages in their viral life cycles. It follows then that what we were measuring by IF and qPCR were both replication competent and incompetent virions that differed little in their binding and entry capability.

Influenza virus can enter the endocytic pathway of cells through a variety of mechanisms, including macropinocytosis, calveolar entry, clathrin-mediated endocytosis, as well as a non-calveolar, non-clathrin dependent pathway (de Vries et al., 2011; Sieczkarski and Whittaker, 2002). Some of these endocytic entry mechanisms have been associated with a need for Sia binding and some lack that requirement (Chu and Whittaker, 2004; de Vries et al., 2012). Out of curiosity, we used both early and late endosomal markers in conjunction with influenza antigen labeling to look at the co-localization of one of the 1918 RBD variants with these
markers and found that it occasionally co-localized but the preponderance of our influenza signal failed to co-localize (Fig. 2). We additionally, incubated our cells with fluorescently conjugated dextran in order to light up the entire endocytic pathway and determine if the influenza antigen is intracytoplasmic but outside the endocytic pathway. However, maintenance of the fluorescent signal throughout the fixation, processing and embedding process has so far proven impossible and alternate techniques working on fixed but unembedded materials are still under development. Most research to date on influenza endocytic entry mechanisms has focused on A/WSN/33 (H1N1), a laboratory mouse-adapted strain and immortalized cell lines. Research into influenza endocytic entry mechanisms using human and avian relevant strains with different HA receptor binding domains and an NHBE model, which is more closely correlated with human respiratory epithelium, may reveal new findings regarding the interplay of RBD and host cells.

Finally, we wondered if there was any correlation between binding and entry ability and replication competence in our viruses. We confirmed that A/SC/1/1918 (SC), A/NY/1/1918 (NY) and the ‘avianized’ 1918 virus (AV) were all multi-cycle replication competent in the NHBE cells (Fig. 3). Interestingly, AV again outperformed its peers. It had a significantly higher titer than SC and NY at the 24 and 36 hr time-points (Fig. 3). We attributed this to its ability to bind and enter the cells quicker and more thoroughly within the given experimental context.
We have shown that all five 1918 RBD variants readily infect NHBE cells (Fig. 3) and that the ‘avianized’ 1918 was the best at binding and entering the NHBE cells at an MOI of 0.01 and a 5 min incubation time (Fig. 5). None of the viruses demonstrated a cell type preference, entering both goblet and ciliated cells quite readily. If anything, viral entry into ciliated cells was slightly delayed for all the viruses. Our work raises many more questions than it answers regarding the importance of sialic acids as a receptor and viral preference for α2,3 vs. α2,6 Sia. However, our findings are in good company with other research, which will hopefully shift the line of inquiry to the investigation of the role of sialic acid in other stages of the viral life cycle including its transmission as well as encourage investigation of alternative influenza receptors.
Acknowledgements

I thank my co-authors on the manuscript based on this work that is currently in preparation: Daniel Chertow, Li Qi, Louis Schwartzman, Jon Suzich, Carolea Logun, James H. Shelhamer and Jeffery K. Taubenberger. Dr. Chertow will be acknowledged co-first with me on this.

The authors would like to acknowledge the staff at the NIAID RTB Bioimaging Section for their assistance with confocal microscopy and image post-processing. We thank Sandra Horton and her staff at the North Carolina State University College of Veterinary Medicine Histopathology Laboratory for their preparation of tissue and embedded NHBE cell sections for immunofluorescence and histological analysis. This work was supported by the intramural grant of NIAID, NIH. ASD and JKT are further thankful for the support of the NIH Comparative Molecular Pathology Research Training Program. This work was done in partial fulfillment of A. Sally Davis’ dissertation work towards a PhD in Comparative Biomedical Sciences at the North Carolina State University College of Veterinary Medicine.
Tables

Table 1: 1918 Pandemic Influenza HA Receptor Binding Domain Variants Amino Acid Sequences*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Abbreviation</th>
<th>Residue at HA1 domain</th>
<th>Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/SC/1/1918</td>
<td>SC</td>
<td>D Q D</td>
<td>α2,6</td>
</tr>
<tr>
<td>A/NY/1/1918</td>
<td>NY</td>
<td>D Q G</td>
<td>α2,3 &gt; α2,6</td>
</tr>
<tr>
<td>“Avianized” 1918</td>
<td>AV</td>
<td>E Q G</td>
<td>α2,3</td>
</tr>
<tr>
<td>A/NY/3/1918</td>
<td>NY3</td>
<td>D Q N</td>
<td>unknown</td>
</tr>
<tr>
<td>A/VA/1/1918</td>
<td>VA</td>
<td>D R D</td>
<td>α2,6 (modeled)</td>
</tr>
</tbody>
</table>

*Sources of data are (Glaser et al., 2005; Reid et al., 1999; Sheng et al., 2011; Stevens et al., 2006; Tumpey et al., 2007).
Table 2: Hemagglutination assay results for AV, NY, and SC

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titer</th>
<th>HA unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>$7.6 \times 10^7$</td>
<td>1:256</td>
</tr>
<tr>
<td>NY</td>
<td>$8.0 \times 10^7$</td>
<td>1:256</td>
</tr>
<tr>
<td>AV</td>
<td>$2.0 \times 10^7$</td>
<td>1:64</td>
</tr>
</tbody>
</table>
Figure 1 Comparison of influenza antigen distribution by cell type for 1918 receptor binding domain variants

Leica SP5 white light laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin embedded NHBE cells. All imaging was done with a 63x oil objective at zoom 1, 1024 x 1024 pixels. The pseudo-colors are influenza antigen (green), goblet cells (magenta), ciliated cells (red), basal cells (blue), and nuclei (gray). There is one virus per row: (1) AV, (2) SC, (3) NY, (4) NY3, and (5) VA; all were 5 min incubations with virus at MOI 0.01. Column 1 is influenza antigen and nuclei only. Column 2 is influenza antigen, all cell type markers and nuclei. Column 3 is the differential interference contrast slice. Column 4 is a merge of the DIC with the fluorescence with the nuclei subtracted. Clearly the influenza antigen signal in SC is less than the other viruses and AV has the strongest signal. Bar 20 µm.
Figure 2 Endosomal markers with influenza antigen

Leica SP5 white light laser confocal maximum projection images of full thickness z-stacks of immunofluorescence labeled formalin-fixed paraffin embedded NHBE cells. All imaging was done with a 63x oil objective at zoom 3.5, 1024 x 1024 pixels. All images have nuclei (grey): (A) is influenza antigen (green), (B) is Rab5 (red), (C) is Lamp1 (magenta) and (D) is the fluorescence merge. Yellow or orange indicates co-localization of influenza antigen with the early endosomal marker Rab5. White indicates co-localization of influenza antigen with late endosomal marker LAMP1. Bar 5 μm.
Figure 3 Replications kinetics for viruses AV, NY and SC

Plaque forming units at 12-, 24- and 36-hours post-infection with MOI 0.1 of NY, SC and AV. (*) denotes that AV is significantly different than both NY and SC at both 24 and 36 hours post-viral infection by ANOVA with an alpha of 0.05.
Figure 4 Real-time PCR results for all five viruses at a multiplicity of infection of 0.1

Real-time PCR data for MOI 0.1 both 5 min (left side) and 20 min (right side) post-viral infection. The data is presented as GAPDH cycle time/MATRIX cycle time. No significant differences were seen between any of the viruses at either time-point.
Figure 5 Real-time PCR results for all five viruses at a multiplicity of infection of 0.01

Real-time PCR data for MOI 0.1 both 5 min (left side) and 20 min (right side) post-viral infection. The data is presented as GAPDH cycle time/MATRIX cycle time. No significant differences were seen between any of the viruses at either time-point. (*) denotes where AV is significantly different from each of the other viruses at the 5 min time-point only by ANOVA with an alpha of 0.01.
References


Chan RW, Chan MC, Nicholls JM & Malik Peiris JS (2013) Use of ex vivo and in vitro cultures of the human respiratory tract to study the tropism and host responses of highly pathogenic avian influenza A (H5N1) and other influenza viruses. Virus Res 178:133-145

Chan RW, Yuen KM, Yu WC, Ho CC, Nicholls JM, Peiris JS & Chan MC (2010) Influenza H5N1 and H1N1 virus replication and innate immune responses in bronchial epithelial cells are influenced by the state of differentiation. PLoS One 5:e8713


Kogure T, Suzuki T, Takahashi T, Miyamoto D, Hidari KI, Guo CT, Ito T, Kawaoka Y & Suzuki Y (2006) Human trachea primary epithelial cells express both sialyl(alpha2-
3) gal receptor for human parainfluenza virus type 1 and avian influenza viruses, and sialyl(alpha2-6)gal receptor for human influenza viruses. Glycoconj J 23:101-106


CHAPTER 5

Conclusion
I set out to improve experimental models for the study of human influenza A pathogenesis and demonstrate use of these models within this new context. I examined the binding and entry of human influenza A viruses in both archival tissue and a primary cell line in new ways that revealed new data that contributes to our understanding of human influenza A pathogenicity, specifically the reconstructed 1918 pandemic influenza virus.

First, I characterized autofluorescence in formalin-fixed, paraffin-embedded human respiratory tissue by measuring it with a new technique and modeling it mathematically. I then used this approach to determine which treatments successfully diminished autofluorescence in this tissue type. Additionally, I tested this technique on other tissue types and tissues preserved by other techniques and found the approach to be generally useful. In fact, since this work has been presented at conferences and published in the Journal of Histochemistry and Cytochemistry, I have been called upon to consult on autofluorescence issues at multiple institutions and found even broader applications for these techniques.

Second, I better characterized the \textit{in vitro} differentiated normal human bronchial epithelial (NHBE) cell model for analysis of human influenza infections. My work provides a side-by-side comparison of NHBEs to their source tissue (human trachea and carina), including cell type presence and distribution, epithelial morphology comparison, influenza A receptor distribution, viral binding and infection pattern comparisons. This is useful because it provides a to date unavailable summary of the strengths and weaknesses of this \textit{in vitro} system such that future research based on this model can be assessed within this deeper context.
Finally, I used this model to analyze the binding and entry of five 1918 pandemic influenza A viruses that differed only in their hemagglutinin receptor binding domain. Contrary to expectations, all 5 viruses bound and entered the cells and the ‘avianized’ virus was the most effective, easily entering both goblet and ciliated cells, including cells that express only $\alpha_{2,6}$ sialic acid (Sia) on their surface. This runs counter to the mainstream dogma that sialic acid distribution controls viral binding and entry based on cell surface glycan distribution and raises a number of interesting new questions regarding the role of sialic acid in influenza A pathogenicity and transmission.

In each chapter discussion, the findings were reported in the context of existing influenza research. I presented both limitations, means to address them and other logical lines of future enquiry. These include further application of the novel techniques developed for the examination of autofluorescence, use of the techniques employed to characterize the NHBEs in the characterization of other viral research models, and further investigation of each of the questions raised by the 5 1918s RBD binding and entry study.

As stated in the introduction, prevailing hypotheses postulate that the hemagglutinin protein of avian-adapted influenza viruses binds host cells via $\alpha_{2,3}$ Sia and that mammalian-adapted viruses bind $\alpha_{2,6}$, and that host switch requires a complete change in this Sia preference. My work failed to support this hypothesis. It showed instead that at least in the context of the NHBE in vitro model, whose epithelial cells, both goblet and ciliated display predominantly $\alpha_{2,6}$ Sia, that entry into cells by an “avianized” virus that binds $\alpha_{2,3}$ glycans exclusively was
not only plausible but more efficient. It led to higher virus content bound to and within the cells and an early lead in viral replication competence compared with an α2,6 exclusive binding preference and an α2,3/α2,6 mixed binding preference virus. While not the first demonstration that Sia is not the only potential receptor of import, it is additional evidence that perhaps the role of Sia may be more complex than simply a determinant of influenza A binding and entry.

In summary, my research has advanced the field of influenza biology by both conducting novel hypothesis driven research that explores the binding and entry of a unique set of 1918 H1N1 receptor binding domain variants and by providing a rich context for the interpretation of these results through my detailed characterization of the NHBE model. Additionally, I have advanced our understanding of autofluorescence in formalin-fixed tissue, providing both a set of guidelines for diminishing this in human respiratory tissue and a general set of tools for tackling this issue in other targets.