Measurement of the Airway Surface Liquid Volume with Simple Light Refraction Microscopy

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In the cystic fibrosis (CF) lung, the airway surface liquid (ASL) volume is depleted, impairing mucus clearance from the lung and leading to chronic airway infection and obstruction. Several therapeutics have been developed that aim to restore normal airway surface hydration to the CF airway, yet preclinical evaluation of these agents is hindered by the paucity of methods available to directly measure the ASL. Therefore, we sought to develop a straightforward approach to measure the ASL volume that would serve as the basis for a standardized method to assess mucosal hydration using readily available resources. Primary human bronchial epithelial (HBE) cells cultured at an air–liquid interface develop a liquid meniscus at the edge of the culture. We hypothesized that the size of the fluid meniscus is determined by the ASL volume, and could be measured as an index of the epithelial surface hydration status. A simple method was developed to measure the volume of fluid present in meniscus by imaging the refraction of light at the ASL interface with the culture wall using low-magnification microscopy. Using this method, we found that primary CF HBE cells had a reduced ASL volume compared with non-CF HBE cells, and that known modulators of ASL volume caused the predicted responses. Thus, we have demonstrated that this method can detect physiologically relevant changes in the ASL volume, and propose that this novel approach may be used to rapidly assess the effects of airway hydration therapies in high-throughput screening assays.

Keywords: airway surface liquid; cystic fibrosis; airway epithelium; epithelial sodium channel; cystic fibrosis transmembrane regulator

Mucociliary clearance, the primary innate defense mechanism of the conducting airways, is strongly influenced by the hydration state of the airway lumen (1). In cystic fibrosis (CF) lung disease, the clearance of mucus and inhaled pathogens is impaired due to an inadequate volume of airway surface liquid (ASL), resulting in mucus obstruction and chronic airway infection (2–4). Therefore, over the last decade, a plethora of therapeutic agents have been developed to block Na^+ absorption (5–10), augment Cl^- secretion (11–16), or osmotically expand the ASL volume (17–20) in an effort to restore proper hydration to the airway surface. Well differentiated primary human bronchial epithelial (HBE) cultures have provided an excellent model to study the physiological processes that underlie mucosal hydration and investigate the effects of therapeutic agents. However, standardized methods to study directly the effects of these compounds on the ASL are lacking.

CLINICAL RELEVANCE

Dehydration of the airway surfaces in cystic fibrosis impairs mucociliary clearance from the lung and numerous therapies to augment the airway surface liquid volume await preclinical evaluation. This manuscript provides a simple method that can be applied to high-throughput measurement of airway mucosal hydration.

Airway epithelia are relatively permeable to water, and, therefore, fluid flux across the airway is believed to be driven by osmotic gradients established by active ion transport (2, 21, 22). In non-CF epithelia, Na^+ absorption through epithelial Na^+ channel (ENaC) increases under conditions when the ASL volume is high, and ASL absorption increases accordingly (23–25). Conversely, when the ASL volume is depleted, Na^+ absorption is inhibited and Cl^- secretion increases, allowing for net secretion of ASL to maintain mucosal hydration. Whereas this dynamic system maintains an optimal ASL volume in healthy HBE, airway epithelia from patients with CF have an impaired ability to secrete Cl^-, due to mutant CF transmembrane conductance regulator (CFTR), and display unregulated Na^+ absorption though ENaC; this leads to excessive ASL absorption and mucosal dehydration (2, 22, 26). Based on these properties, it is believed that the changes in the short circuit current (I_{SC}) across HBE predict changes in airway surface hydration. However, there are several factors that may limit the ability of I_{SC} to predict changes in the ASL volume. Ussing chamber measurements are performed with extremely large/fixed volumes of fluid and pharmacological agents, relative to what is normally experienced by the airways. Due to these nonphysiological conditions, I_{SC} measurements do not take into account (*1*) the effects of soluble endogenous regulators of airway ion transport/ASL flux and (*2*) the ability of the airways to spontaneously remove/metabolize pharmacological compounds that have been added to the mucosal surface (23–25, 27). Therefore, a drug/therapy may not have the same effect *in vivo* that was observed in the Ussing chamber. In addition, I_{SC} measurements fail to account for changes in paracellular permeability and the potential contribution of nonelectrogenic ion transport. Therefore, it is critical that the ASL is measured directly to understand the effects of therapeutic agents on mucosal hydration.

The ASL height can be directly measured using confocal microscopy (2, 3). In these assays, a small bolus of fluid, containing a nonabsorbable, fluorescently labeled dextran is applied to the apical surface of HBE cultures and then the height of this liquid layer is serially measured over time. ASL height measurements have provided a direct means to investigate ASL regulation, and have dramatically increased our knowledge of basic CF pathophysiology. However, these methods require sophisticated equipment, and can only be performed in specialized laboratories that have experience performing this tech-

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nique. Therefore, we sought to develop simple methodology to measure the ASL volume using readily available resources. Primary HBE cells cultured at an air–liquid interface (ALI) typically develop a liquid meniscus at the edge of the culture insert, as demonstrated in Figure 1A. We reasoned that the size of the fluid meniscus is likely determined by the ASL volume, and could be measured as an index of the epithelial surface hydration status. To establish the relationship between the meniscus and the ASL volume, a series of experiments was performed using primary HBE cultured on an ALI. First, we directly imaged the meniscus using XZ confocal imaging and established the relationship between the ASL volume, ASL height, and size of the meniscus. Next, we developed a simple method to measure the ASL volume by imaging the light refraction caused the fluid meniscus that is formed between the thin film ASL and the vertical wall of the culture insert (*see* Figure 1B). Using this novel approach, we compared the ASL volume of HBE cultured from CF and non-CF tissue donors and examined the effects of hypertonicity and ion channel blockade on ASL volume.

MATERIALS AND METHODS

Primary Human Airway Epithelial Cell Culture

HBE cells were cultured from excess pathological tissue after lung transplantation and organ donation under protocols approved by the Universities of Pittsburgh and North Carolina investigational review boards, as previously described (24). Cells were studied after 3–6 weeks of culture, and considered differentiated when a mucociliary phenotype was apparent on phase-contrast microscopy.

Confocal Measurement of ASL Height and ASL Volume

HBE cultures were grown under air–liquid conditions on 12-mm-diameter Transwell clear membranes (Corning, Lowell, MA) until fully differentiated, as previously described (25). Immediately before measuring the ASL, cultures were washed three times with PBS to remove excess mucus. Rhodamine-dextran (10 kD, 2 mg/ml) was added to the final wash, and the PBS was aspirated with a Pasteur pipette until ASL height was at minimal levels (22, 25, 28). Cultures were then placed in a chamber on the stage of an inverted Lecia SP5 confocal microscope. ASL height was then measured using a 63 × 1.2 NA glycerol immersion lens, as previously described (22, 25, 28). To obtain images of the ASL meniscus, cultures were scanned in XZ mode using a 20×, 0.7 NA, or a 10×, 0.4 NA dry, objective lens. The cross-sectional image of the meniscus was obtained from four different regions at the perimeter of the culture (corresponding to 0°, 90°, 180°, and 270°). The cross-sectional surface area of each meniscus was measured using ImageJ (29). Because the confocal microscope laser beam intensity diminishes with distances greater than approximately 30 μm in fluid, the laser power and gain were increased to the point that the region of the ASL image nearest the laser beam was saturating to visualize the portion of the meniscus at the ALI. However, because the meniscus follows a uniform path, when the regions of interest were drawn, obvious image blurring and saturation were ignored. To obtain the meniscus volume (V_{men}; *see* Figure 1B), the meniscus was assumed to be cylindrically symmetric. Thus, V_{men} was calculated by multiplying the average of the four cross-sectional surface areas of the meniscus (0°, 90°, 180°, and 270°) by the circumference of the meniscus (πd). The remainder of the ASL volume covering the thin film liquid present across the ALI (V_{ALI}) was calculated by multiplying the ASL height by πr^2, assuming this volume was a cylinder. The total ASL volume was then calculated as the sum of $V_{men} + V_{ALI}$.

Measurement of ASL Volume Using Refracted Light Microscopy

HBE cultures grown in a 24-well plate on 0.33-cm^2 filter inserts were visualized with a standard cell culture microscope (CK40; Olympus, Center Valley, PA) with a 5× plain light objective (Zeiss, Thornwood, NY) and imaged using a Scion CFW 1610M camera with μManager (30). Images of the meniscus were taken at the culture perimeter at 90° or 270° without phase contrast. A custom algorithm was developed using ImageJ (NIH, Bethesda, MD) (29) to automate the analysis of the meniscus as discussed in the results section.

RESULTS

The Fluid Meniscus Surrounding HBE Cultures Reflects the ASL Volume

During the initial development of confocal ASL height measurements, the ASL height increment for a given volume of apical fluid was less than predicted (2). This discrepancy was attributed to accumulation of fluid within a meniscus at the edge between the culture and the Transwell wall. However, the partitioning of the ASL between the meniscus and the thin film ASL was not elucidated. Therefore, we developed a method to image the ASL height across a large surface area, which allows for calculation of the ASL volume contained both within the fluid meniscus (V_{men}) and across the surface of the HBE culture grown on the ALI (V_{ALI}). Accumulated mucus was removed by several washes with PBS and the ASL labeled with rhodamine-dextran. The apical surface was subsequently aspirated to

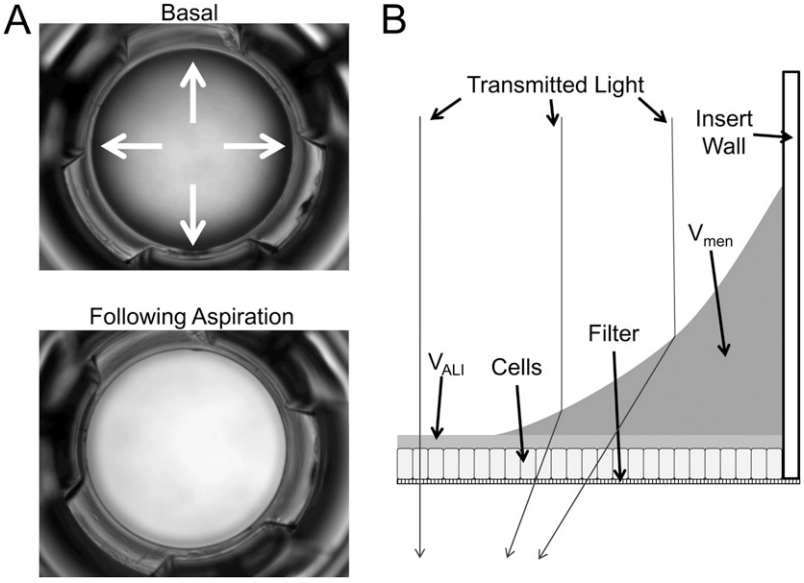

Figure 1. The fluid meniscus at the human bronchial epithelial (HBE) culture perimeter. (*A*) Low-power image depicting the fluid meniscus that surrounds primary HBE cultures. As shown in the representative photomicrographs, the Transwell containing the epithelial cells is surrounded by a fluid meniscus (*arrows*). After aspiration of the airway surface liquid (ASL) and mucus, the fluid meniscus is no longer present. (*B*) Schematic demonstrating the shape of the meniscus from an XZ perspective. The thin-film ASL volume present over the HBE at an air–liquid interface (V_{ALI}, *light gray*) and the volume present in the meniscus (V_{men}, *dark gray*) are labeled, along with the cell culture insert wall, HBE cells, and permeable culture filter. Light refraction of the transmitted light induced by the curving ALI at the meniscus, as shown by the *vertical arrows*, causes the graded light intensity across the meniscus.

set the ASL height at minimal levels (22, 25). As shown in the low-magnification images of the meniscus and in the higher-magnification thin-film images of ASL height in Figure 2A (top panel and bottom panel, respectively), a small volume of fluid remains after aspiration. From these images, the V_{men} was calculated to be 2.1 (±0.3) μl compared with 1.5 (±0.2) μl in the V_{ALI}. Therefore, 3.6 (±0.2) μl of ASL remained present on the apical surface of 12-mm HBE cultures after aspiration, which is likely the result of a small residual volume that cannot be aspirated from the apical surface after labeling with the fluorescent fluid.

Next, we determined the distribution patterns of the ASL as the volume was experimentally expanded. As shown in Figure 2B, the V_{men} and V_{ALI} were measured after the serial addition of 5-μl boluses of PBS. There was excellent correlation with the total measured ASL volume and the actual volume added plus the residual volume that could not be aspirated (R^2 = 0.98; slope = 1.2 ± 0.04; y intercept = 3.6 ± 0.4). During the titration, V_{ALI} doubled, but, as clearly seen in Figure 2B, the bulk of the volume added went to the meniscus and little went into V_{ALI}. This behavior is consistent with the contact angle of the meniscus being zero at the interface with the thin film ASL and nonzero at the interface with the culture insert wall, which is made of polystyrene and is therefore somewhat hydrophobic. As the ASL volume increased, the meniscus between these two surfaces enlarged; extending both across the thin-film ASL and up the side wall, an action that would continue until the menisci from diametrically opposed positions on the insert meet. Presumably, at this point, any additional fluid would raise the height of the ASL in the center of the cell.

We then compared the kinetics of ASL volume absorption and ASL height regulation after an apical fluid challenge. As shown in Figure 2C, 15 μl of fluorescently labeled PBS was applied to the apical surface of differentiated HBE cultures and the height of the thin-film ASL on the HBE cells and the total ASL volume were serially measured over a 48-hour period. As previously shown, the thin-film ASL height rapidly returned to a basal height of approximately 10 μm, suggesting that the ASL height is acutely regulated by HBE cultures. Conversely, the ASL volume required a longer period of time to return to basal levels, due to the accumulation of fluid in the meniscus. These results suggest that the ASL height in close proximity to the cells and the total ASL volume are differentially regulated; whereas the thin film ASL height is acutely determined by active ion transport, the ASL volume present in the meniscus appears to serve as a reservoir compartment that requires an extended period of time to return to basal levels.

Development of a Simple Method to Measure the ASL Volume

Because the bulk of the total ASL volume is present within the meniscus surrounding the HBE culture insert, we sought to develop a simple method of assessing the ASL volume by imaging the meniscus. When examined with a standard cell culture microscope under low power, the fluid meniscus appears as a gradation of gray scales that are generated by the refraction of light as it passes though the sloping liquid layer, diagramed schematically in Figure 1B. In the representative photomicrograph in Figure 3, the wall of the culture insert is oriented to the right side of the image. Adjacent to the wall, the dark meniscus begins and gradually fades as the liquid meniscus ends on the flat thin film ASL. An image of the meniscus is generated by the refraction of the light at the meniscus interface away from the incident direction and the collection objective. The greater the angle of the air–liquid interface, the greater the angle of refracted light, and the darker the image. Therefore, the image of the meniscus is determined by the shape of the meniscus and, as such, reflects the volume and surface tension of the ASL contained within the culture insert.

To standardize the quantification of the meniscus image, we developed a custom script using the open-source ImageJ software. To remove noise and obtain a representative profile of the gray levels generated by the meniscus, the gray level is averaged down a line of 200 pixels oriented perpendicular to the radial direction of the cell culture insert. This averaged gray level is then plotted versus the radial distance, as shown in Figures 3A and 3B. The gray level array is then truncated at the

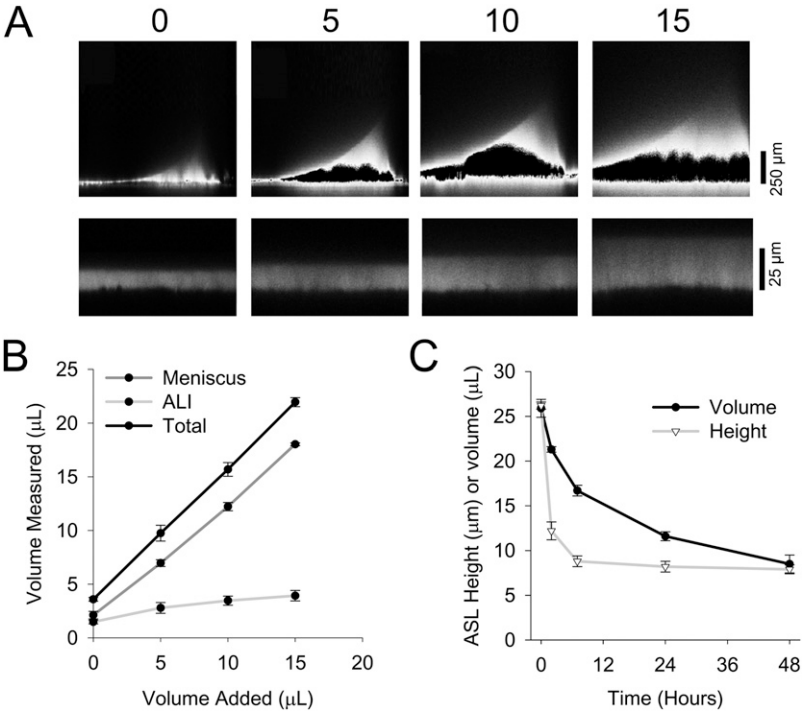

Figure 2. Direct measurements of ASL volume by XZ-confocal microscopy. (*A*) Typical images of the ASL meniscus, labeled with rhodamine-dextran, which occurs at the interface between the cell culture periphery and the tissue culture insert wall. (*B*) Mean calculated ASL volume after sequential 5 μl additions of PBS to the mucosal surface. During this experiment, all PBS contained 100 μM amiloride to block Na^+ and fluid absorption. Data shown are mean V_{men} (meniscus), V_{ALI} (ALI), and total ASL volume (n = 3 cultures). (*C*) Mean ASL height and ASL volume after the apical addition of 15 μl PBS. Data shown are mean ASL height or volume (n = 6 cultures).

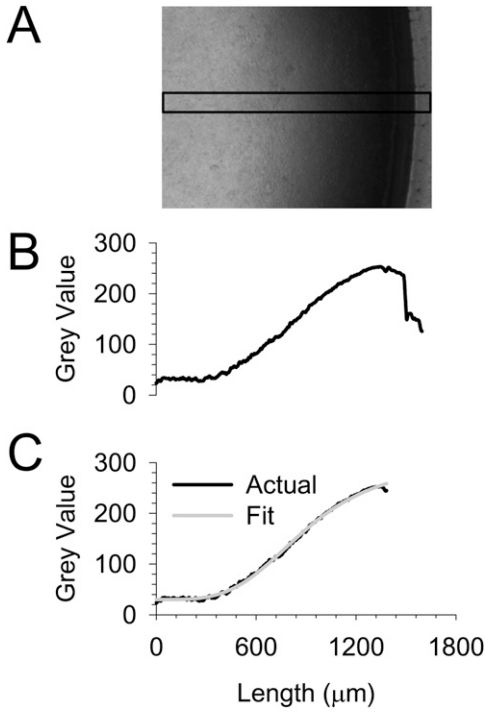

Figure 3. Quantification of the HBE culture meniscus using low-power light refraction microscopy. Primary HBE cultures were placed on an inverted stage of a cell culture microscope and the Transwell perimeter was imaged with a 5× objective. (*A*) Representative photomicrograph demonstrating the Transwell insert wall (*right*) adjacent to the fluid meniscus that surrounds the HBE culture. The 200-pixel-high region of interest used for the automated analysis is shown by the *black box*. (*B*) Raw light intensity across the meniscus. The intensity array is truncated when the light intensity begins to decline to remove the insert wall from the analysis. (C) Comparison of the actual (Actual, *black line*) intensity profile and the derived (Fit, *gray line*) intensity profile from the sigmoidal fit.

point at which the value begins to drop below the peak value, as shown in Figure 3C (black trace), to remove the culture insert wall from the analysis. The truncated array (Grey value versus length) is then fit to a four-parameter logistic function where:

$$\text{Grey value} = \text{min} + (\text{max} - \text{min})/1 + (\text{x}/\text{EC50})^{\text{Hillslope}})$$

When fit to this equation, the R^2 values are typically greater than 0.99, indicating an excellent fit. The length of the meniscus can be determined as the length from where the Grey value is greater than *min* to the end of the array and the area under the curve (AUC) is measured using the numerically integrated rectangular rule. Therefore, the meniscus length, AUC, and several parameters describing the meniscus shape can be mathematically derived from the image of the meniscus using the automated image analysis algorithm.

Due to the complexity of the image formation as the transmitted light passes through both the meniscus and culture insert, the intensity profile is not a simple XZ representation of the meniscus shape. Therefore, we experimentally determined the correlation between the derived parameters of the intensity profile and the ASL volume, rather than mathematically calculating the volume based on the AUC of the intensity profile. To establish the relationship between the refracted light image of the meniscus and the ASL volume, the native ASL was aspirated from the apical surface of differentiated HBE cultures and the meniscus was imaged after serial additions of 2.5 μl PBS to the

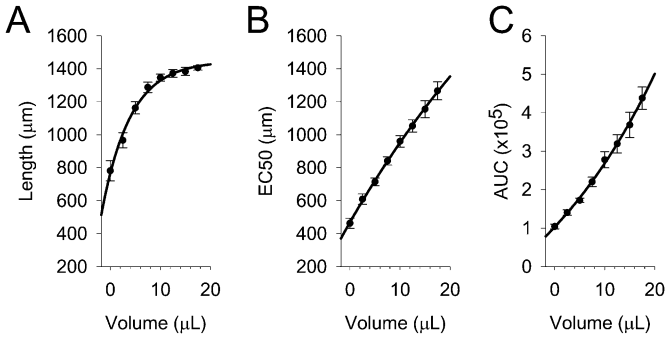

Figure 4. Calibration of the HBE culture meniscus with the ASL volume. The apical surfaces of differentiated HBE cultures were aspirated and subsequently used for volume titrations. Increments of PBS (2.5 μl) were applied to the apical surface and the ASL meniscus was measured. Data shown are the mean (±SEM) meniscus length (*A*), distance from the culture insert wall to half of the grey value between max and min (EC50) (*B*), and area under the curve (AUC [C]) for the HBE cultures with increasing ASL volume (n = 12 cultures from 2 tissue donors).

apical surface. As shown in Figure 4, the meniscus length, EC50, and AUC demonstrated a dose response to increasing ASL volume. Due to limitations in the field of view and the length of the mensicus, we were unable to completely visualize the meniscus with volumes greater than 10 μl. Because of the limited field of view, the meniscus length reached a plateau when the ASL volume exceeded 10 μl, because the light intensity did not drop below the *min* parameter from the sigmoidal fit. However, the EC50 and AUC continued to increase with increasing volumes allowing for an ASL volume resolution of approximately 0–20 μl. Because the EC50 measurements correlated well with the ASL volume over a wide range, we used this value to calculate the ASL volume for the remainder of the experiments. Thus, the ASL volume can be measured from the image of the meniscus by integrating the EC50 value with the experimentally derived calibration curve shown in Figure 4B.

We next examined the effect of various potential confounding factors on the ASL volume measurement. First, we found that it is important to orient the intensity profile so that it is perpendicular to a tangent of perimeter of the cell culture insert. Therefore, by convention, all images of the meniscus were acquired at 90° or 270° with respect to the culture insert, allowing for automated analysis. As shown in Figure 5, the measured ASL volume for an individual filter was unchanged by the ambient light in the room. Furthermore, the light intensity of the microscope had little effect on the measured ASL volume, provided that the light intensity of the image was not over or under saturated. The ASL volume measurement was not significantly altered by the focal plane at which the images were obtained. The best measurements were obtained when the meniscus was imaged without phase contrast, because the phase contrast introduced ring artifacts. Likewise, the intensity profile quality suffered from noise introduced by the culture insert wall, when the focal plane was above the filter. Based on the robust measurement of the ASL volume, the images can be rapidly obtained with a low-NA objective at a focal plane slightly below the filter, without the need for frequent refocusing or adjustment of the light intensity.

HBE Cells Cultured from CF Donors Have a Decreased ASL Volume Compared with Non-CF Cells

The lack of functional CFTR in CF lung disease leads to defective fluid secretion and excessive fluid absorption from the luminal airway surface of CF HBE (2, 26). As previous studies

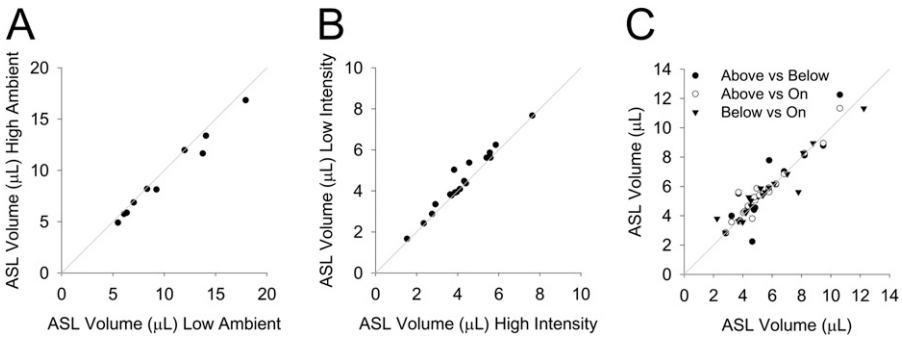

Figure 5. Contribution of light intensity and focal plane on the ASL volume measurement. The ASL volume of HBE cultures with varying ASL volumes were sequentially measured before and after changes in ambient light surrounding the microscope, illumination intensity, and focal plane during image acquisition. Data shown are the comparison between low and high ambient light (*A*), low and high illumination intensity (*B*), and fine focus adjusted above, on, or below the HBE culture (*C*) ($n \geq 10$ cultures for each comparison). A line is plotted with a slope of 1 for reference (*gray line*).

have indicated a decreased ASL height in CF, we hypothesized that the ASL volume of CF HBE would also be decreased with respect to non-CF tissue. The ASL volumes of HBE cultured from several tissue donors containing a variety of CFTR genotypes were measured under baseline conditions with no prior manipulation to the native ASL, as shown in Figure 6. Overall, HBE cultured from CF tissue donors had an average ASL volume of 1.5 (±0.2) μl versus 6.5 (±0.9) μl in non-CF tissues ($P < 0.001$ by Mann-Whitney rank sum; $n = 11$ tissue donors). Aside from one donor with an R117H/dF CFTR genotype, HBE cultured from CF donors had less than 2 μl of ASL. Interestingly, the ASL volume of the CF HBE cultures containing one copy of a mild mutation (R117H/dF CFTR) (31) had an intermediate ASL volume between the severe CFTR mutations and wild-type genotypes. The non-CF HBE cultures had a more variable ASL volume with respect to the uniformly low volume present on the CF cultures. Therefore, CF HBE cultures have a reduced ASL volume and the refracted light from the meniscus method of measuring the volume is sensitive enough to detect this disease-relevant difference.

ASL Volume Response to Apical Tonicity and Ion Channel Blockers

To define the strengths and limitations of the ASL volume method of assessing the airway surface hydration status, we next examined the ASL volume response to manipulations with predictable effects. First, we examined the effect of hypertonicity on CF HBE cultures, as hypertonic saline and mannitol have previously been shown to increase the ASL. The ASL volume of differentiated CF HBE cells was expanded with 5 μl PBS (±300) mM mannitol, and the ASL volume was measured at 0, 4, 8, 24, 48, and 72 hours after the fluid bolus. As shown in Figure 7, the cultures that were treated with the hypertonic fluid had a more pronounced and prolonged ASL volume expansion compared with the filters exposed to isotonic fluid. Therefore, using this simple method of assessing the ASL volume, we are able to measure changes in the ASL volume in response to apical fluid boluses and obtain the predicted response to hypertonic fluids.

We next determined whether we could measure changes in the ASL volume in response to inhibition of the basolateral sodium potassium chloride cotransporter with bumetanide. Because this drug would be expected to block active chloride secretion from HBE cultures, we anticipated that the ASL volume would be decreased after treatment with bumetanide. Differentiated HBE cultures were cultured in the presence or absence of 100 μM bumetanide for 3 days before measuring the ASL volume. As shown in Figure 8, the ASL volume decreased to 1.1 ± 0.1 μl in the bumetanide-treated cultures, compared with 10.6 (±1.0) μl in the control filters ($P < 0.001$; $n = 15$ cultures derived from 3 different tissue donors). These results confirm that ASL volume is actively regulated and dependent on Cl^- secretion.

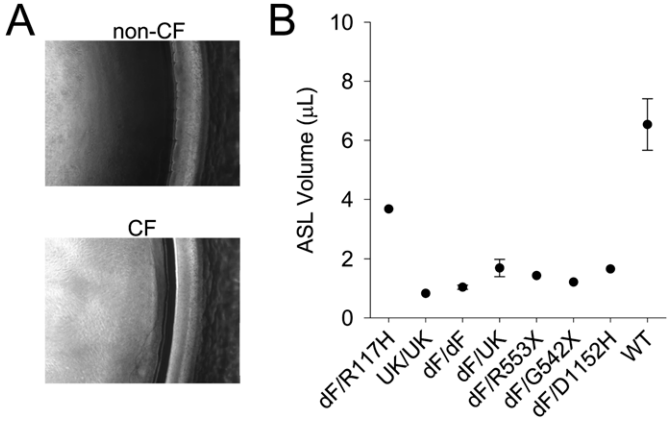

Figure 6. Comparison of the ASL volume of HBE cultures with various cystic fibrosis (CF) transmembrane conductance regulator (CFTR) genotypes. The ASL volume of several CF and non-CF HBE cultures was measured. (*A*) Representative photomicrographs of the meniscus present on CF and non-CF HBE cultures. (*B*) Mean ASL volume for each tissue donor arranged by CFTR genotype ($n = 11$ CF and 11 non-CF tissue donors).

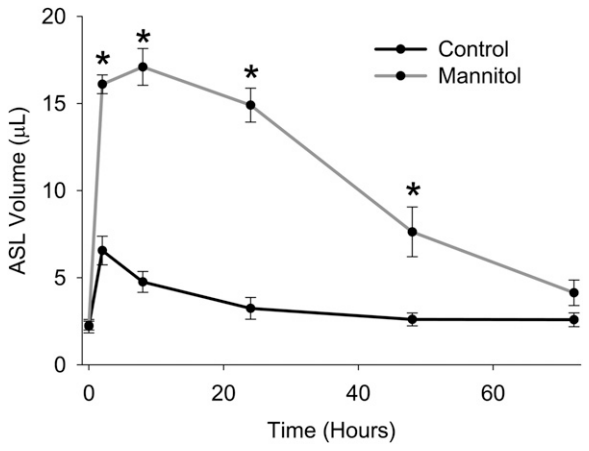

Figure 7. Comparison of the ASL volume after expansion with isotonic versus hypertonic fluid. The ASL volume of differentiated HBE cultures was measured at 0, 2, 4, 8, and 24 hours after ASL volume expansion with Ringer's solution with or without 300 mM mannitol. Data shown are mean ASL volume (±SEM) ($n = 18$ cultures from 3 different tissue donors).

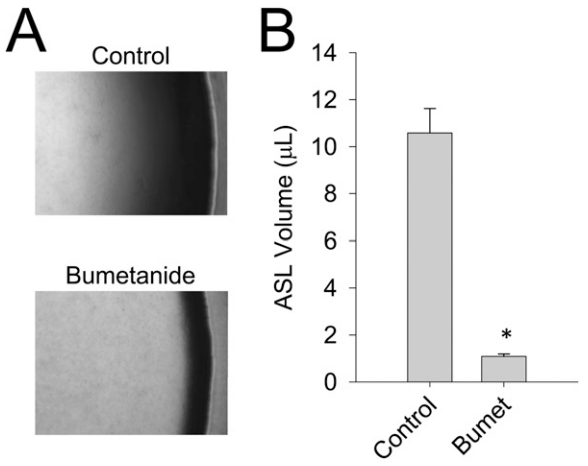

Figure 8. The ASL volume is dependent on chloride secretion. Bumetanide (100 μM) was added to the basolateral media of differentiated HBE cultures for 3 days before measurement of the ASL volume. (A) Representative photomicrograph of the meniscus under control and bumetanide-treated conditions. (*B*) Mean (±SEM) ASL volume of HBE cultures with or without 100 μM bumetanide ($n = 16$ cultures from 3 tissue donors). *$P < 0.001$ ASL height is different from control.

DISCUSSION

Adequate hydration of the airway surface is critical to efficient mucociliary clearance in the lung, and, as such, numerous therapies have been developed to increase the ASL volume in CF airways. Given the limited number of patients with CF and resources available to perform clinical studies of these novel therapeutic agents, extensive preclinical evaluation is required to prioritize which agents merit expedited investigation. Primary cultures of HBE have provided an excellent model system for the study of airway physiology and response to therapy; to date, the electrophysiological studies performed on primary HBE cultures have accurately predicted *in vivo* nasal potential differences (12, 32–35). In the current manuscript, we describe a simple method to directly measure the ASL volume of cultured HBE that can be applied to the preclinical evaluation of airway hydration agents as an additional outcome measure. Because the bulk of the ASL volume is contained within the fluid meniscus that surrounds the culture well, the meniscus can be used as a marker of the apical volume present on air–liquid HBE cultures. Using conventional low-magnification light microscopy, we developed a method to measure the ASL volume based on the light refraction pattern induced as the transmitted light passes through the meniscus. As anticipated, the ASL volume was dependent on CFTR genotype, and HBE cultured from CF donors had a significant reduction in the amount of fluid present on the apical surface. When Cl^- secretion was inhibited using bumetanide, the ASL volume fell to levels comparable to those found in CF cultures. Furthermore, we examined the effect of hypertonic fluid on the ASL, which produced a large and durable expansion of the ASL volume similar to what has been observed in patients with CF who inhale hypertonic saline (17, 18). Therefore, the ASL volume can be readily measured using light microscopy, and is a relevant marker of mucosal hydration in cultured HBE cells.

Because of the difficulties associated with measuring the height of the roughly 10-μm-thin film ASL, the most commonly used method to predict changes in the airway surface hydration is to measure I_{SC}, and assume that a decrease in Na^+ absorption or an increase in Cl^- secretion will augment the ASL volume. However, this method is indirect, and is performed under conditions that substantially alter HBE physiology (23–25). I_{SC} measurement also fails to include the potential contribution of nonelectrogenic ion transport and paracellular permeability. Therefore, I_{SC} data may not always accurately predict the effect of a given intervention on mucosal hydration, as demonstrated by the lack of benefit when amiloride is combined with hypertonic saline therapy in patients with CF (17). The gold standard assessment of the ASL in cultured epithelia to date has been to measure its height using confocal microscopy. This technique requires expensive confocal microscopy and considerable technical skill to perform; as such, confocal height measurements are time consuming and not readily suitable for high-throughput assays. In addition, as the native ASL is manipulated and fluorescently labeled before ASL height measurement, this approach places the HBE cultures at risk for contamination. Measuring the ASL volume using refracted light microscopy avoids these pitfalls, as it does not require prior manipulation of the cultures, and uses widely available, inexpensive imaging systems to directly measure the ASL volume.

Measuring the ASL volume using refracted light microscopy is dependent on the development of a meniscus at an artificial interface between the thin-film ASL and the culture insert wall. Because this interface is not present in the native airway, it is difficult to draw direct physiological correlation between the meniscus compartment and *in vivo* conditions present within the airway lumen. Therefore, the ASL volume measured in cultured HBE cells does not convey the same physiological implications that the ASL height measurements provide. Because outstretched cilia are approximately 7-μm tall and mucus clearance is impaired when the ASL height decreases below the height of the cilia, it is reasonable to infer that interventions that augment the ASL height toward 7 μm would be sufficient to improve mucociliary clearance from the CF lung (22). Conversely, the ASL volume of cultured epithelia does not directly convey this physiologically relevant information. Another caveat of the ASL volume measurement is the slow rate of volume regulation with respect to the rapid kinetics observed with confocal height measurement. Because the acute physiological processes that determine airway surface hydration can be rapid and transient, confocal height measurements are likely to remain the gold standard assessment of the ASL. Therefore, the ASL volume measurement is most suitable for the assessment of durable changes to the absorptive/secretory properties of HBE, than to manipulations that cause rapid transient changes.

Interestingly, the ASL volume required a prolonged period to return to basal levels after a volume challenge compared with the ASL height (Figure 2C). Because the V_{men} is contiguous with the V_{ALI}, one would anticipate that, as the V_{ALI} is reabsorbed after a volume challenge, the V_{men} would be absorbed with similar kinetics. We propose that the disparity between the V_{ALI} and V_{men} absorption kinetics is due to changes in the proportion of the HBE surface exposed to an expanded ASL height. Immediately after the fluid challenge, the entire epithelial surface is exposed to ASL expansion, which leads to ENaC activation across the entire culture. At this early time point, the epithelium assumes an absorptive phenotype and ASL absorption is maximal. As the excess ASL is absorbed, an increasing portion of the epithelial surface returns to basal ASL height and, as such, Na^+ and ASL absorption decreases. Because the total ASL absorption rate reflects the net absorption across the entire surface area, the rate of ASL absorption will decrease as a smaller portion of the culture is exposed to ASL expansion.

The contribution of ASL surface tension on the meniscus shape was not resolved in the current study. Because the shape

of the meniscus is determined by both the volume and surface tension properties of the meniscus, changes in surface tension would likely cause changes in the measured meniscus shape. However, previous studies have indicated that the surface tension of the ASL is approximately 34 mN/m, and that this low tension persists when isotonic fluid is added in excess to the apical culture surface. To appreciably change the ASL surface tension, the surface active materials and mucus had to be removed with DTT-containing PBS (22). Based on this previous work, it is unlikely that the surface tension properties of the ASL vary significantly during the experiments performed in this manuscript. However, it is possible that the shape of the meniscus would be altered by interventions that effect mucus rheology and/or surface tension properties. Further studies are required to determine the effects of surface tension and mucus composition on the meniscus shape.

Despite the caveats of measuring the ASL volume by imaging the meniscus, this is a straightforward approach to measure directly the ASL volume of cultured HBE without the need for sophisticated equipment or prior manipulation of the native ASL. Theoretically, the best data from the meniscus are obtained using a low-NA objective and unfocused transmitted light. These optical configurations allow for the capture of an optical image that conveys information regarding the contour of the meniscus without artifact introduced from the culture structure or cells. Fortunately, these conditions are present on inexpensive cell culture microscopes fitted with low-power objectives. To implement this technique, we recommend using a non–phase-contrast low-power objective that will provide a field of view greater than the radius of the cell culture insert, to image the entire meniscus. This approach is limited at high volumes, when the diametrically opposed menisci combine without separation by the thin-film ASL, because an intervening plateau at the center of the image is required to accurately define the shape of the meniscus. However, this limitation is unlikely to be significant, because this would only occur at apical volumes far greater than what is typically observed by HBE cultures. Because the optical configurations may differ, a calibration curve between the meniscus shape and ASL volume should be performed with any new system. Due to the undemanding optical requirements and wide volume range, high-throughput analysis of the ASL volume could be developed using a microscope fitted with a mechanical stage, an optical plate reader, or a top illumination scanner.

In summary, we have developed a rapid assay to assess the ASL volume using plain light microscopy to image the pattern of light refracted by the meniscus surrounding primary airway epithelial cultures. The ASL volume measurement is not dependent on fine focus, and the analysis is automated; as such, the ASL volume can be rapidly measured and the results are highly objective and reproducible. In addition, the method can be used as an added outcome for any experiment that may have an effect of mucosal hydration, as the cells can subsequently be used for further study with no risk of contamination or effect from the ASL volume measurement. Therefore, this method is suitable for high-throughput screening of candidate therapeutic agents to augment the airway surface hydration in CF.

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