Novel Stereological Methods for Alveolar Cell Quantification in the Mouse Lung

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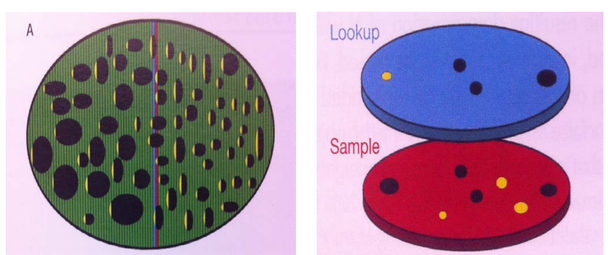
Abstract

Understanding whether lung development occurs by increase in cell size or cell number can lead to relevant clinical implications in phenomena such catch up growth, chronic neonatal lung disease and cancer. Although alveolar type 1 (AT1) and type 2 (AT2) cells have been quantified in the past, these methods are not up to par with current software and 3D molecular imaging. Here, novel stereological methods are proposed for alveolar cell quantification in the murine lung. Lungs were harvested from postnatal FVB Wild Type Mice at two time points: days 2 (P2) and 37 (P37). Volumes were measured using two different methods: fluid displacement and Cavalieri Principle, the latter with the use of the Stepanizer® software. Immunofluorescence staining with ECAD and HOPX using whole mount staining led to visualization of 3D structures using confocal microscopy. These images were used to quantify and calculate the numerical density of AT1 and AT2 in the Imaris® software. With the application of the Cavalieri Principle, the total absolute number of AT1 and AT2 cells was analyzed at both time points of postnatal lung development. When comparing P2 and P37, we found that the left lung was 19 uL and 98 uL, respectively. The numerical density of alveolar cells for left lung in a P2 was 188.6 AT1 cells/3.0 x 10^6 um^3 and 166.6 AT2 cells/3.0 x 10^6 um^3, and in a P37 was 280.75 AT1 cells/6.0 x 10^6 um^3 and 724.1 AT2 cells/6.0 x 10^6 um^3. A total number alveolar cells in a P2 were 1.11 x 10^6 AT1 cells and 9.83 x 10^5 AT2 cells, and in a P37 were 4.5 x 10^6 AT1 cells and 1.18 x 10^7 AT2 cells. These results suggest that lung development occurs by an increase in cell size over time.

Introduction

Stereology is a branch of science focused in the study of three-dimensional objects in two-dimensional sections with the application of mathematical analysis (Weibel 1979; West, 2012). The stereological probes used for the volume measurements are the disector, area, lines and points. Object number is obtained with the use of a disector and data cannot be derived solely on a two-dimensional section if morphologic information about the object in study is not provided (West, 2012).



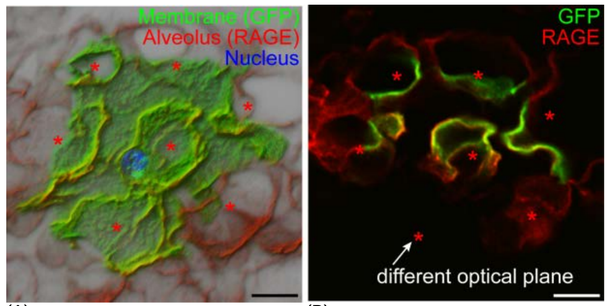
(A) (B)

Figure 1. Counting methods in stereology. (A) The leading edges of cells within a tissue section are marked in yellow. (B) The Disector Method compares 2 sequential sections to see the appearance and the disappearance of the cell. (Taken from West, 2012).

Introduction (continued)

In this study, lung volume is estimated through two different methods: fluid displacement and the Cavalieri Principle. The calculated total lung volume can then be used for the elucidation of the murine total alveolar number of cells, in particular squamous alveolar type 1 (AT1) and surfactant-producing alveolar type 2 (AT2) cells.

The most effective and unbiased method for quantification of object number is the 3D probe named the disector (**Figure 1b and 4**). The disector counting technique allows the counting of objects in an unbiased manner in a 3D volume produced by two sequential sections either physical or optical **(Figure 1a**) (Sterio, 1984; Kaplan et al., 2012). Nevertheless, AT1 cells have never been quantified before in an unbiased and accurate manner, because there were not target-specific molecular markers. Recently, a new research study confirmed that AT1 cells expressed a new marker, named HOPX, in the nucleus and membrane of these cells (**Figure 2**) (Barkauskas et al. 2013).



(A) (B)

Figure 2. (A) Genetically labeling of AT1 cell spans multiple alveoli in 3D, whereas in (B) AT1 cell in 2D looks fragmented.

Crapo and colleagues have used stereological methods for the quantification of alveolar cells in the past in 1982, but the electromicrographs were taken from a single section. ***Thus, we designed a new unbiased method for alveolar cell quantification using 3D molecular imaging*** (**Figures 3-4**)**.**

Whether lung development occurs by increase in cell size or cell number can lead to relevant clinical implications such as catch up growth, chronic neonatal lung disease and cancer.

Hypothesis

Lung development occurs by an increase of cell size rather than by cell number.

Methods

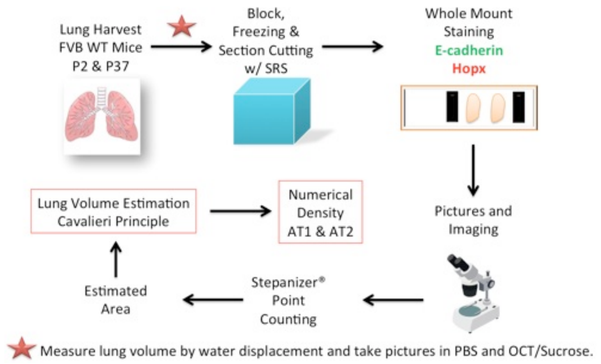


Figure 3. Summary of the proposed general method for the quantification of AT1 and AT2 cells using 3D Molecular Imaging. (SRS = Systematic Random Sampling).

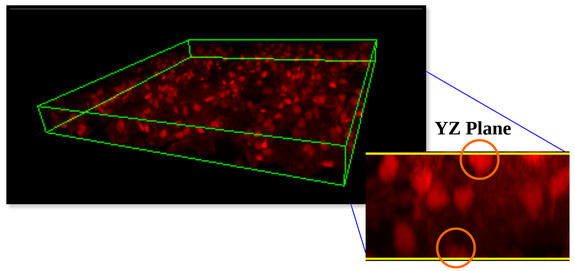
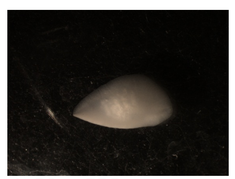
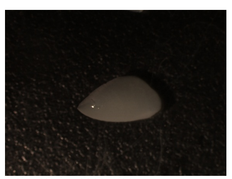


Figure 4. Disector method using 3D imaging. The leading edges can be determined easily using the Imaris® software, and we can appreciate the cell appearing and disappearing in a 3D structure.

Results

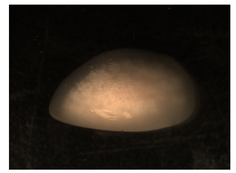


(A) P2 in PBS

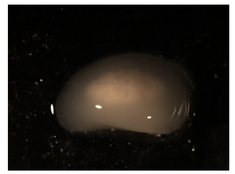


(B) P2 in OCT/Sucrose

Results (continued)



(C) P37 in PBS



(D) P37 in OCT/Sucrose

Figure 5. Visual comparison of left lobes of P2 and P37 after Phosphate Buffered Saline (PBS) solution 1X and Optimum Cutting Temperature Formulation with Sucrose 30% (OCT/Sucrose) overnight incubation. Picture dimensions: 2048 x 1536 pixels.

Table 1. Numerical density (N_v) of AT1 and AT2 cells in FVB Wild Type Mouse P2 and P37.

FVB Wild Type	N_v AT1 Cells	N_v AT2 Cells
P2	188.6 cells/3.0 x 10^6 um^3	166.6 cells/3.0 x 10^6 um^3
P37	280.75 cells/6.0 x 10^6 um^3	724.1 cells/6.0 x 10^6 um^3

Table 2. Total absolute number (N) of AT1 and AT2 cells in FVB Wild Type Mice P2 and P37.

FVB Wild Type	N AT1 Cells	N AT2 Cells
P2	1.11 x 10^6 cells	9.83 x 10^5 cells
P37	4.5 x 10^6 cells	1.18 x 10^7 cells

Conclusions

The data obtained for numerical density and total absolute number of AT1 and AT2 cells suggest that lung development of a FVB WT mouse occurs by an increase of cell size rather than by cell number. If lung development were to occur by an increase in cell number, the numerical density would have been at least a three-fold increase within the reference volume for a P37. As well, the total absolute number of alveolar cells would at least be a three-fold increase in terms of potency when comparing a P2 with a P37. Further research is required to elucidate alveolar cell number within the postnatal days 2 and 37 in order to confirm these results.

References

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