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BACHELOR THESIS IN BIOMEDICAL ENGINEERING

DESIGN OF LUNG IN VITRO MODELS

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ABSTRACT

"Organs-on-chips are micro-engineered biomimetic systems containing microfluidic channels lined by living human cells, which replicate key functional units of living organs to reconstitute integrated human organ-level pathophysiology in vitro." These microdevices can be used to test efficacy and toxicity of drugs and chemicals, thus creating *in vitro* models of human disease. Besides, they potentially represent low-cost alternatives to conventional animal models for pharmaceutical, chemical and environmental applications. Organs-on-chips allows to recapitulate tissue-tissue interfaces, along with complex organs-specific chemical and mechanical microenvironments, to mimic key 3D functional units of living human organs.

In this project, we will apply the principles defined for the creation of organs-on-chips, to study the main lung-on-chip models, defining the characteristics along with their advantages and disadvantages of models already proposed in different studies. To create a 'breathing' lung-on-a-chip that mimics the mechanically active alveolar-capillary interface of the living human lung, human alveolar epithelial cells and microvascular endothelial cells were cultured in a microdevice with physiological flow and cyclic suction applied to the side chambers to reproduce rhythmic breathing movements.

Finally, we will propose from a theorical point of view a method of manufacturing lungs-on-chips in a laboratory, summarizing recent progress and the key challenges expected in the near future.

Key words: Organs-on-chip; microfluidic channels; in vitro; microdevices

RESUMEN

"Los órganos en chips son sistemas biomiméticos de microingeniería que contienen canales microfluídicos revestidos por células humanas vivas, que replican unidades funcionales clave de órganos vivos para reconstituir la fisiopatología integrada a nivel de órganos humanos in vitro". Estos microdispositivos pueden usarse para probar la eficacia y la toxicidad de medicamentos y productos químicos, y para crear modelos in vitro de enfermedades humanas. Además, potencialmente representan alternativas de bajo coste a los modelos de animales convencionales para aplicaciones farmacéuticas, químicas y ambientales. Los órganos en chips permiten recapitular las interfaces tejido-tejido, junto con microambientes químicos y mecánicos complejos específicos de órganos, para imitar unidades funcionales clave en 3D de órganos humanos vivos.

En este trabajo, aplicaremos los principios definidos para la creación de órganos en chips, para estudiar los principales modelos de pulmones en chip, definiendo las características junto con sus ventajas y desventajas de modelos ya planteados en distintos estudios. Para crear un pulmón en chip que imite la interfaz alveolar-capilar mecánicamente activa del pulmón humano vivo, las células epiteliales alveolares humanas y las células endoteliales microvasculares se cultivaron en un microdispositivo con flujo fisiológico y succión cíclica aplicadas en cámaras laterales para reproducir movimientos de respiración rítmicos.

Por último, desde un punto de vista teórico se propondrá un método de fabricación en laboratorio de estos pulmones en chip, resumiendo el progreso reciente y los desafíos clave que se esperan en un futuro próximo.

Palabras clave: Órganos en chip; canales microfluídicos; in vitro; microdispositivos

SOMMARIO

“Gli organi su chip sono sistemi biomimetici di microingegneria che contengono canali microfluidici rivestiti da cellule umane viventi, che replicano le unità funzionali chiave degli organi viventi per ricostituire la fisiopatologia integrata a livello di organo umano in vitro." Questi microdispositivi possono essere utilizzati per testare l'efficacia e la tossicità di farmaci e sostanze chimiche e per creare modelli in vitro di malattie umane. Inoltre, rappresentano alternative a basso costo ai modelli animali convenzionali per applicazioni farmaceutiche, chimiche e ambientali. Gli organi su chip consentono la ricreazione delle interfacce tessuto-tessuto, insieme a complessi microambienti chimici e meccanici specifici per organo, per imitare le unità funzionali 3D chiave degli organi umani viventi.

In questo lavoro applicheremo i principi definiti per la creazione di organi su chip, per studiare i principali modelli di polmoni su chip, definendo le caratteristiche insieme ai loro vantaggi e svantaggi dei modelli già proposti in diversi studi. Per creare un polmone su chip che imita l'interfaccia alveolare-capillare meccanicamente attiva del polmone umano vivente, le cellule epiteliali alveolari umane e le cellule endoteliali microvascolari sono state coltivate in un microdispositivo con flusso fisiologico e aspirazione ciclica applicati nelle camere laterali per riprodurre i movimenti respiratori.

Infine, da un punto di vista teorico, verrà proposto un metodo di fabbricazione in laboratorio per questi polmoni a chip, che riassume i recenti progressi e le principali sfide attese nel prossimo futuro.

Parole chiave: Organi su chip; canali microfluidici; in vitro: microdispositivi

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Objectives

The main objectives of this project are:

- Explain the main characteristics of the Organs-on-a-chip, focusing on lungs-on-a-chip.
- Expose the main elements for the creation of lungs-on-a-chip.
- Define a laboratory manufacturing protocol from a theorical point of view.

With these objectives, what is intended is to know what Organs-on-chips are, as well as their main characteristics and what is expected of them in the future.

1. Introduction

The cells of the body need energy for all their metabolic activities. Most of this energy is derived from chemical reactions, which can only take place in the presence of oxygen (O_2). The main waste product of these reactions is carbon dioxide (CO_2). The respiratory system provides the route by which the supply of oxygen present in the atmospheric air enters the body, and it provides the route of excretion for carbon dioxide. [1]

The condition of the atmospheric air entering the body varies considerably according to the external environment, e.g. it may be dry or moist, warm or cold, and carry varying quantities of pollutants, dust or dirt. As the air breathed in moves through the air passages to reach the lungs, it is warmed or cooled to body temperature, saturated with water vapor and 'cleaned' as particles of dust stick to the mucus which coats the lining membrane. Blood provides the transport system for O_2 and CO_2 between the lungs and the cells of the body. Exchange of gases between the blood and the lungs is called *external respiration* and that between the blood and the cells *internal respiration*. The organs of the respiratory system are: [1] ***(Figure 1)***

- nose
- pharynx
- larynx
- trachea
- two bronchi (one bronchus to each lung)
- bronchioles and smaller air passages
- two lungs and their coverings, the pleura
- muscles of breathing – the intercostal muscles and the diaphragm.

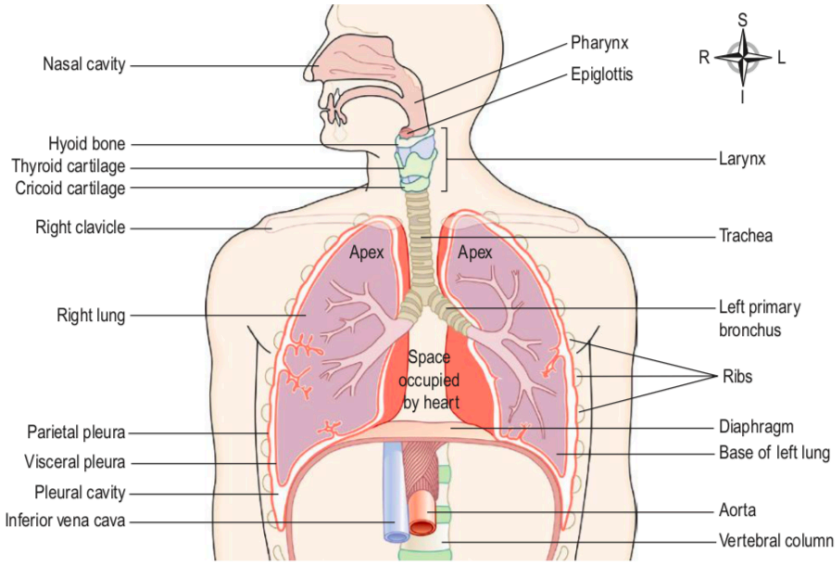


Figure 1. Structures associated with the respiratory system

1.1. Anatomy [1]

We are going to focus on the lower respiratory tract that includes the trachea, bronchi, bronchioles, alveolar sacs and lungs. They will have a completely asymmetric and protected distribution covered by a layer that protects them, the pleura.

1.1.1. The trachea

The trachea or windpipe is a continuation of the larynx and extends downwards to about the level of the 5th thoracic vertebra where it divides at the *carina* into the right and left primary bronchi, one bronchus going to each lung. It is approximately 10–11 cm long and lies mainly in the median plane in front of the esophagus. ***(Figure 2)***

The tracheal wall is composed of three layers of tissue and is held open by between 16 and 20 incomplete (C-shaped) rings of hyaline cartilage lying one above the other. The rings are incomplete posteriorly where the trachea lies against the esophagus. The cartilages are embedded in a sleeve of smooth muscle and connective tissue, which also forms the posterior wall where the rings are incomplete.

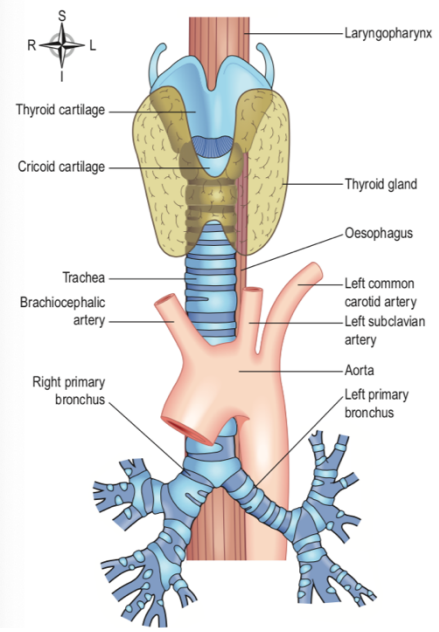


Figure 2. The trachea and some of its related structures

Three layers of tissue 'clothe' the cartilages of the trachea.

- The outer layer contains fibrous and elastic tissue and encloses the cartilages.
- The middle layer consists of cartilages and bands of smooth muscle that wind round the trachea in a helical arrangement. There is some areolar tissue, containing blood and lymph vessels and autonomic nerves. The free ends of the incomplete cartilages are connected by the trachealis muscle, which allows for adjustment of tracheal diameter.
- The lining is ciliated columnar epithelium, containing mucus-secreting goblet cells

1.1.2. Bronchi and bronchioles

The two primary bronchi are formed when the trachea divides, at about the level of the 5th thoracic vertebra.

The right bronchus. This is wider, shorter and more vertical than the left bronchus and is therefore more likely to become obstructed by an inhaled foreign body. It is approximately 2.5 cm long. After entering the right lung at the hilum, it divides into three branches, one to each lobe. Each branch then subdivides into numerous smaller branches.

The left bronchus. This is about 5 cm long and is narrower than the right. After entering the lung at the hilum, it divides into two branches, one to each lobe. Each branch then subdivides into progressively smaller airways within the lung substance.

The bronchial walls contain the same three layers of tissue as the trachea and are lined with ciliated columnar epithelium. The bronchi progressively subdivide into bronchioles, ***(Figure 3)*** terminal bronchioles, respiratory bronchioles, alveolar ducts and finally, alveoli. The wider passages are called *conducting airways* because their function is to bring air into the lungs, and their walls are too thick to permit gas exchange.

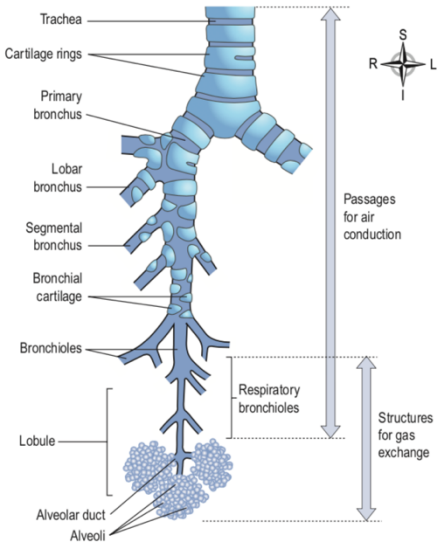


Figure 3. The lower respiratory tract

As the bronchi divide and become progressively smaller, their structure changes to match their function.

Cartilage. Since rigid cartilage would interfere with expansion of lung tissue and the exchange of gases, it is present for support in the larger airways only. The bronchi contain cartilage rings like the trachea, but as the airways divide, these rings become much smaller plates, and at the bronchiolar level there is no cartilage present in the airway walls at all.

Smooth muscle. As the cartilage disappears from airway walls, it is replaced by smooth muscle. This allows the diameter of the airways to be increased or decreased through the influence of the autonomic nervous system, regulating airflow within each lung.

Epithelial lining. The ciliated epithelium is gradually replaced with non-ciliated epithelium, and goblet cells disappear.

1.1.3. Respiratory bronchioles and alveoli

Within each lobe, the lung tissue is further divided by fine sheets of connective tissue into *lobules*. Each lobule is supplied with air by a terminal bronchiole, which further subdivides into respiratory bronchioles, alveolar ducts and large numbers of alveoli (air sacs). It is in these structures that the process of gas exchange occurs.

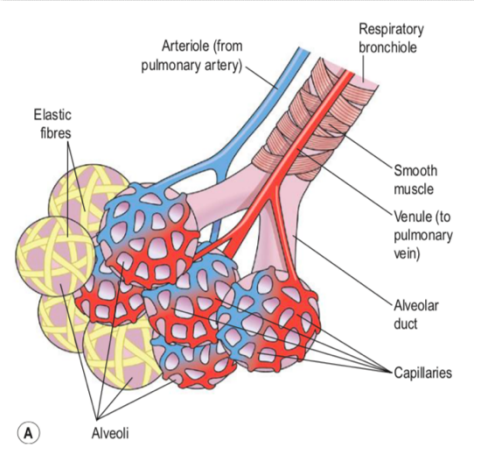


Figure 4. The alveoli and their capillary network

As airways progressively divide and become smaller and smaller, their walls gradually become thinner until muscle and connective tissue disappear, leaving a single layer of simple squamous epithelial cells in the alveolar ducts and alveoli. These distal respiratory passages are supported by a loose network of elastic connective tissue in which macrophages, fibroblasts, nerves and blood and lymph vessels are embedded. The alveoli are surrounded by a dense network of *capillaries* ***(Figure 4)*** *Exchange* of gases in the lung (external respiration) takes place across a membrane made up of the alveolar wall and the capillary wall fused firmly together. This is called the *respiratory membrane*.

1.1.4. Lungs

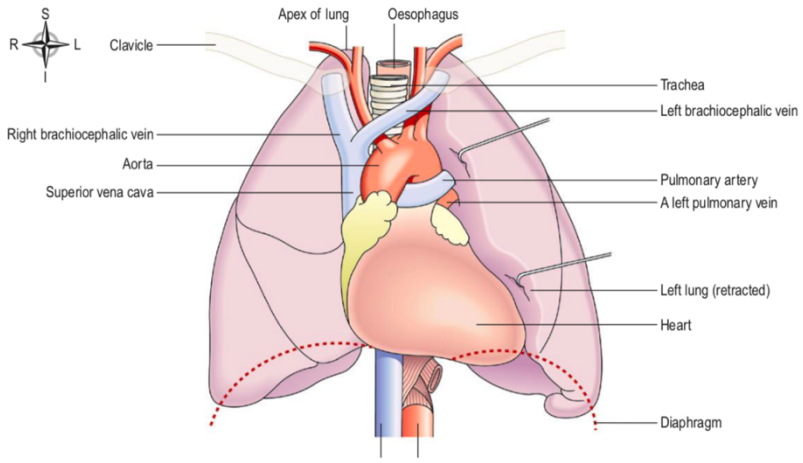


Figure 5. Organs associated with the lungs

There are two lungs, one lying on each side of the midline in the thoracic cavity. They are cone-shaped and have an apex, a base, a tip, costal surface and medial surface. ***(Figure 5)***

<u>The apex</u>: This is rounded and rises into the root of the neck. It lies close to the first rib and the blood vessels and nerves in the root of the neck.

<u>The base:</u> This is concave and semilunar in shape and lies on the upper (thoracic) surface of the diaphragm.

The costal surface: This is the broad outer surface of the lung that lies directly against the costal cartilages, the ribs and the intercostal muscles.

The medial surface: The medial surface of each lung faces the other directly across the space between the lungs, the *mediastinum*. Each is concave and has a roughly triangular-shaped area, called the *hilum*, at the level of the 5th, 6th and 7th thoracic vertebra. The primary bronchus, the pulmonary artery supplying the lung and the two pulmonary veins draining it, the bronchial artery and veins, and the lymphatic and nerve supply enter and leave the lung at the hilum ***(Figure 6).***

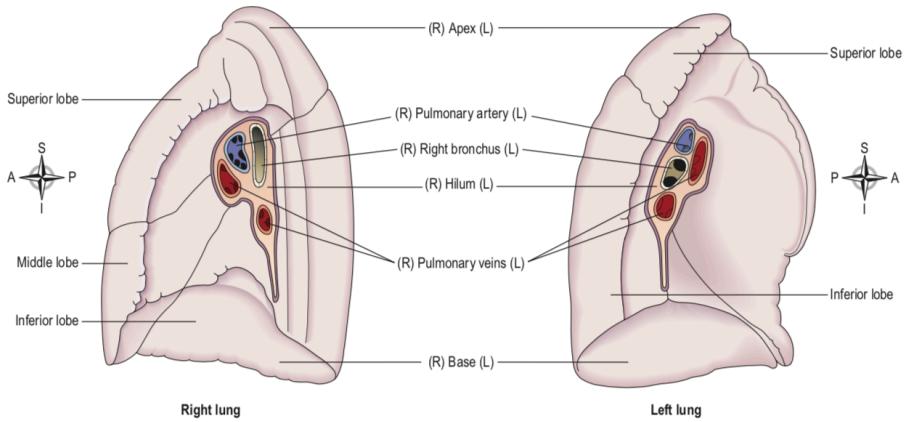


Figure 6. The lobes of the lungs and vessels/airways of each hilium. Medial views

The mediastinum contains the heart, great vessels, trachea, right and left bronchi, esophagus, lymph nodes, lymph vessels and nerves.

The right lung is divided into three distinct lobes: superior, middle and inferior. The left lung is smaller because the heart occupies space left of the midline. It is divided into only two lobes: superior and inferior. The divisions between the lobes are called *fissures.*

Pleura

The pleura consists of a closed sac of serous membrane (one for each lung) which contains a small amount of serous fluid. The lung is pushed into this sac so that it forms two layers: one adheres to the lung and the other to the wall of the thoracic cavity. ***(Figure 7)***

- Visceral pleura: This is adherent to the lung, covering each lobe and passing into the fissures that separate them.
- Parietal pleura: This is adherent to the inside of the chest wall and the thoracic surface of the diaphragm. It is not attached to other structures in the mediastinum and is continuous with the visceral pleura round the edges of the hilum.

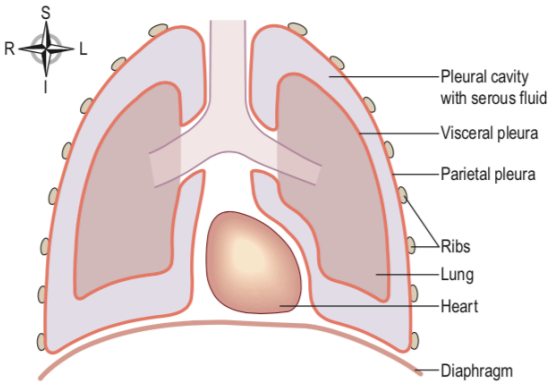


Figure 7. The relationship of the pleura to the lungs

The pleural cavity

This is only a potential space and contains no air, so the pressure within is negative relative to atmospheric pressure. In health, the two layers of pleura are separated by a thin film of serous fluid (pleural fluid), which allows them to glide over each other, preventing friction between them during breathing. The pleural fluid is secreted by the epithelial cells of the membrane.

The two layers of pleura, with pleural fluid between them, behave in the same way as two pieces of glass separated by a thin film of water. They glide over each other easily but can be pulled apart only with difficulty, because of the surface tension between the membranes and the fluid. This is essential for keeping the lung inflated against the inside of the chest wall. The airways and the alveoli of the lungs are embedded in elastic tissue, which constantly pull the lung tissues towards the hilum, but because pleural fluid holds the two pleurae together, the lung remains expanded. If either layer of pleura is punctured, air is sucked into the pleural space and part or all of the entire underlying lung collapses.

Pulmonary blood supply

The pulmonary trunk divides into the right and left pulmonary arteries, carrying deoxygenated blood to each lung. Within the lungs each pulmonary artery divides into many branches, which eventually end in a dense capillary network around the alveoli ***(Figure 8)***. The walls of the alveoli and the capillaries each consist of only one layer of flattened epithelial cells. The exchange of gases between air in the alveoli and blood in the capillaries takes place across these two very fine membranes (together called the *respiratory membrane*). The pulmonary capillaries merge into a network of pulmonary venules, which in turn form two pulmonary veins carrying oxygenated blood from each lung back to the left atrium of the heart.

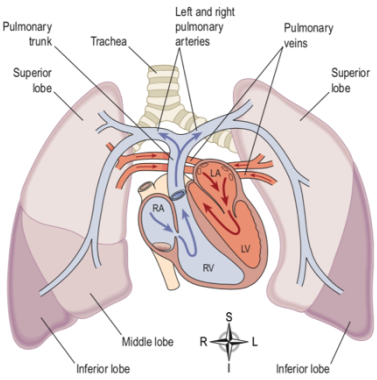


Figure 8. The flow of blood between heart and lungs

1.2. Physiology [2]

The main functions performed by the respiratory system are:

- Gases exchange between the atmosphere and the blood
- Homeostatic regulation of pH
- Protection and defense of the organism
- Vocalization

In physiology we can talk of two types of respiration. On the one hand we have the cellular respiration and on the other hand we have the mechanical respiration. The first one is the interaction between the oxygen and the organic molecules to produce CO_2, water and energy in the ATP form. The second one is the responsible for moving the gases between the environment and the cells of our organism. This movement of air in and out is known as ventilation.

1.2.1. Ventilation

Ventilation is what we commonly called respiration, and it has two main movements: Inspiration, which introduces air in the lungs and expiration that makes the contrary process.

VENTILATION= INSPIRATION + EXPIRATION

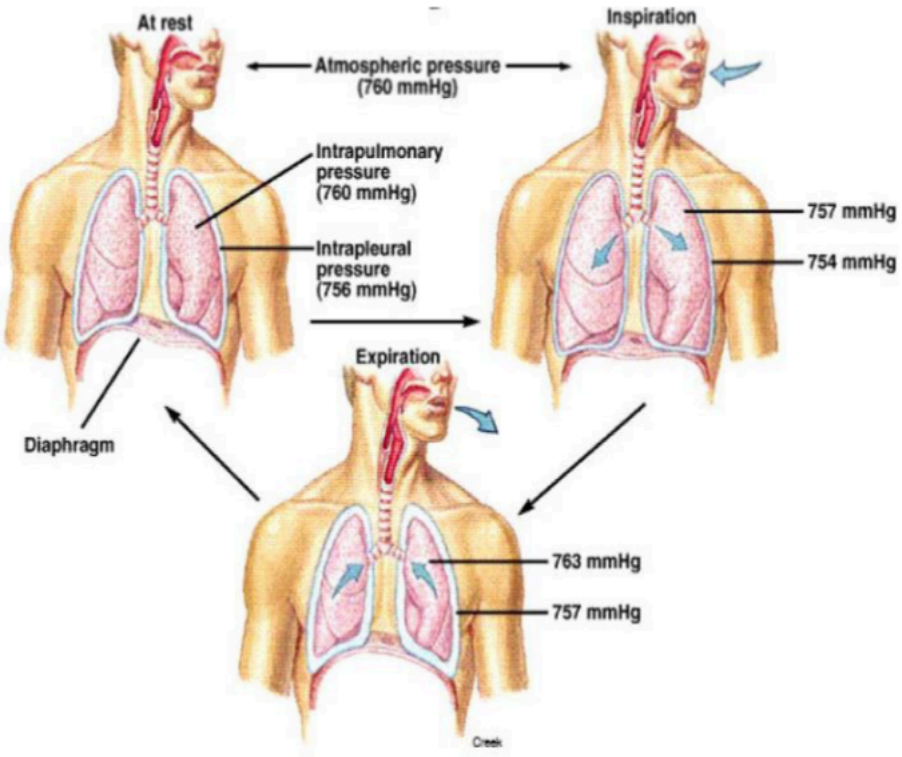


Figure 9. Breathing mechanism

These movements can be carried out thanks to the different respiration pressures.

- Pleural pressure
- Alveolar pressure
- Transpulmonary pressure

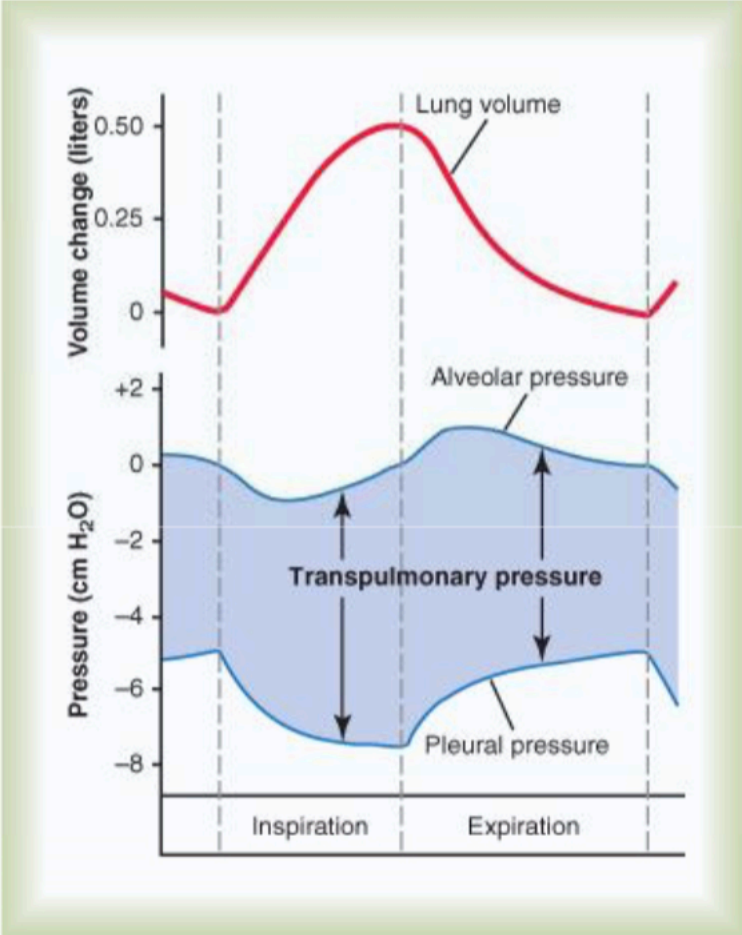


Figure 10. Pressures involved in the respiratory process

1.2.2. Lung volumes and capacities

Another important aspect in physiology is lung volumes and capacities which are typically measure with a spirometer.

- Tidal volume: volume of air that fills the alveoli plus the volume of air that fills the airways.
- Inspiratory reserve volume: additional volume that can be inspired above tidal volume.
- Expiratory reserve volume: additional volume that can be expired below tidal.

- Residual volume: Volume of gas remaining in the lungs after a maximal forced expiration.
- Inspiratory capacity: Composed of the tidal volume plus the inspiratory reserve volume.
- Functional residual capacity: Is composed of the expiratory reserve volume plus the residual volume.
- Vital capacity: Is composed of the inspiratory capacity plus the expiratory reserve volume.
- Total lung capacity: Finally, the TLC includes all of the lung's volumes.

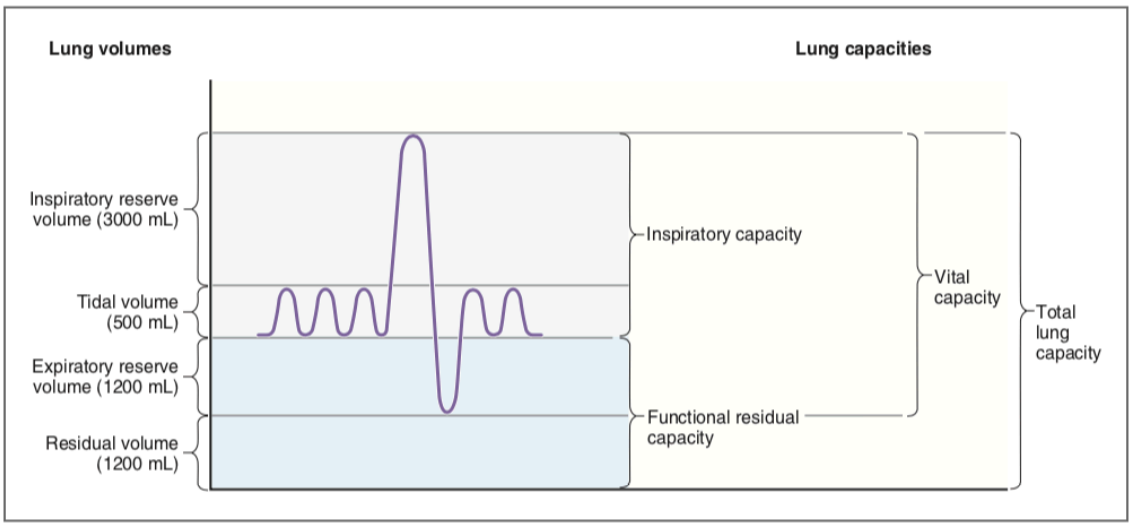


Figure 11. Parameters for lung volumes and capacities

1.2.3. Pulmonary gas exchange

The pulmonary gas exchange takes place between the alveolar air and the blood flow through the pulmonary capillaries

The factors that determine the amount of oxygen that diffuses into the blood are:

- The oxygen pressure gradient that exist between the alveolar air and the blood
- The total functioning surface of the respiratory membrane
- The respiratory minute volume
- Alveolar ventilation

Structural factors that helps the diffusion of the oxygen from the alveolar air into the blood:

- The fact that the alveolar and capillary walls form only a thin barrier for gases to pass through.
- That the alveolar and capillary surfaces are large.

- That the blood circulates through the capillaries in a thin layer, so that each red cell passes very close to the alveolar air.

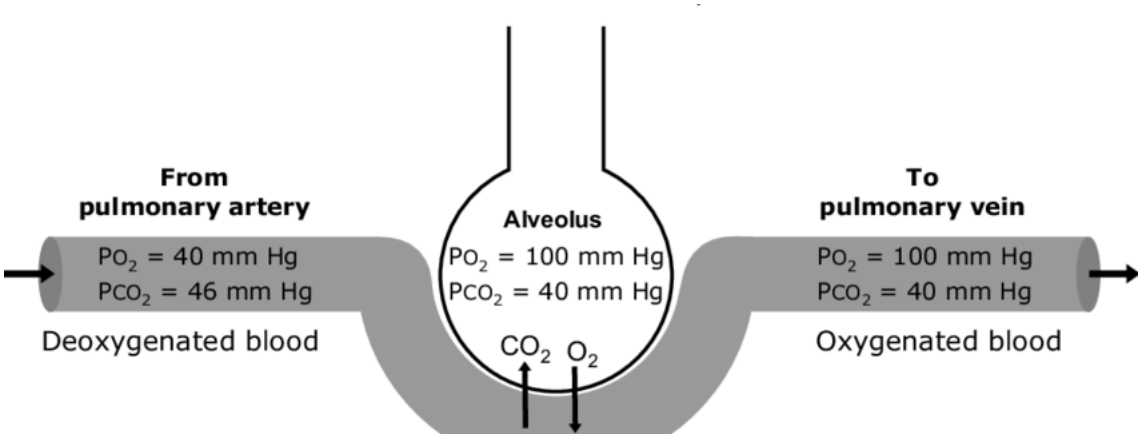


Figure 12. Pulmonary gas exchange. Transfer of O_2 and CO_2 between alveolar air and capillary blood

1.2.4. Respiratory regulation

The main integrative centers that control the nerves that supply the inspiratory and expiratory muscles are located in the brain stem.

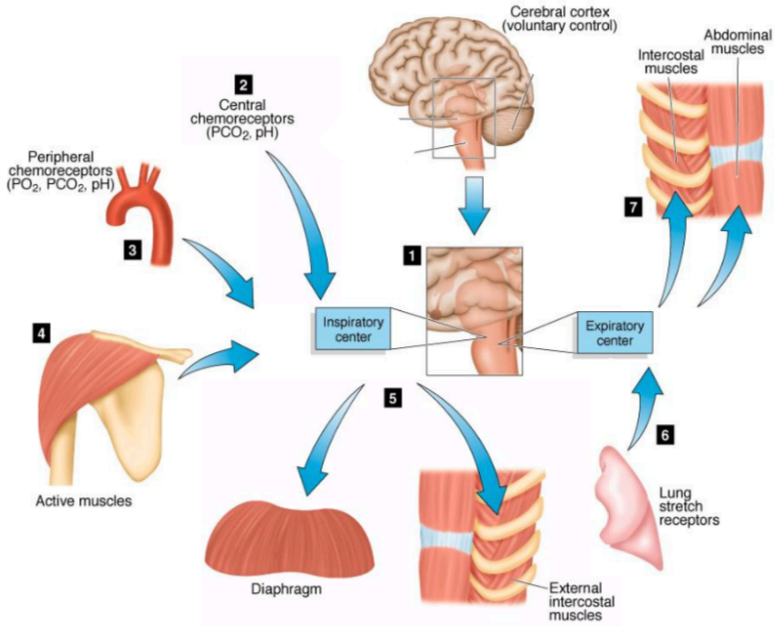


Figure 13. Neural regulation of respiration

1.3. Histology [3]

Specialized cells collaborate to warm, moisturize, and remove particles that enter. These cells are the respiratory epithelium and comprise the entire respiratory tree. The respiratory epithelium is typically a ciliated pseudostratified columnar epithelium with globet cells, which will gradually decrease in height, and it will change from pseudostratified to cylindrical epithelium and cubic epithelium. The number of globet cells will also decrease along the progression. These are the main cells in the respiratory system:

Main cells	Characteristics
Ciliated cells	They are the most abundant. Cylindrical, with eosinophilic cytoplasm and nuclei at different heights. They control the actions of the mucociliarity escalator, a primary defense mechanism that removes debris.
Globet cells	They are cells with calyx morphology, which have the nucleus in a basal position. They have a pale apical cytoplasm. They decrease in number as the respiratory tree gets progressively smaller.
Brush cells	They are cylindrical cells, with microvilli at the apical pole and an eosinophil cytoplasm. Approximately 3%
Basal cells	The basal cells connect to the basement membrane and provide the attachment layer of the ciliated cells and goblet cells. They may be thought of as the stem cells of the respiratory epithelium as they maintain the ability to potentiate ciliated cells and goblet cells
Neuroendocrine cells	They have neurosecretory type granules and can secrete several factors. This includes catecholamine and polypeptide hormones, such as serotonin, calcitonin, and gastrin-releasing factors
Granule cell	They are round cells that appear at different heights of the epithelium with a broad nucleus. They are typically lymphocytes in transit.

Table 1. Main cells of the respiratory epithelium

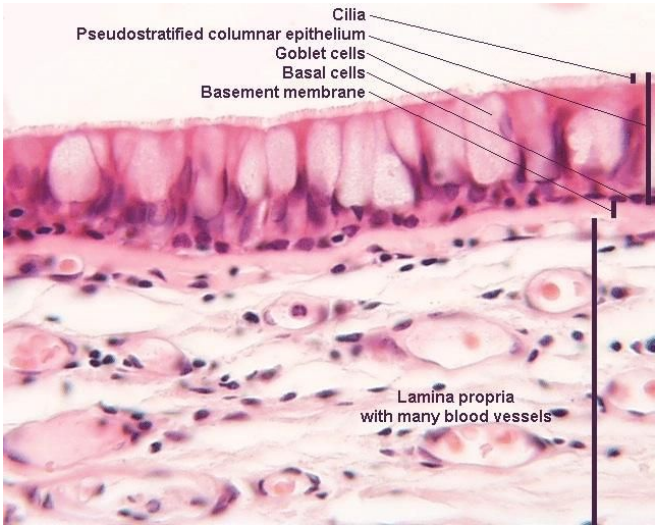


Figure 14. Main cells of the respiratory epithelium

It is important to note that after the basement membrane we have connective tissue. This underlying connective tissue is rich in fibroblasts, collagen fibers, and elastic fibers and in some places host glands. It is known as **lamina propria.**

1.3.1. Respiratory portion

We are going to focus on the cells of the respiratory portion, as they will be of great help to understand the following pages. As we said before, the respiratory portion is made up of the respiratory bronchioles, the alveolar duct and the alveolar sacs. The alveoli appear in these structures and are the functional units that carry out the gas exchange.

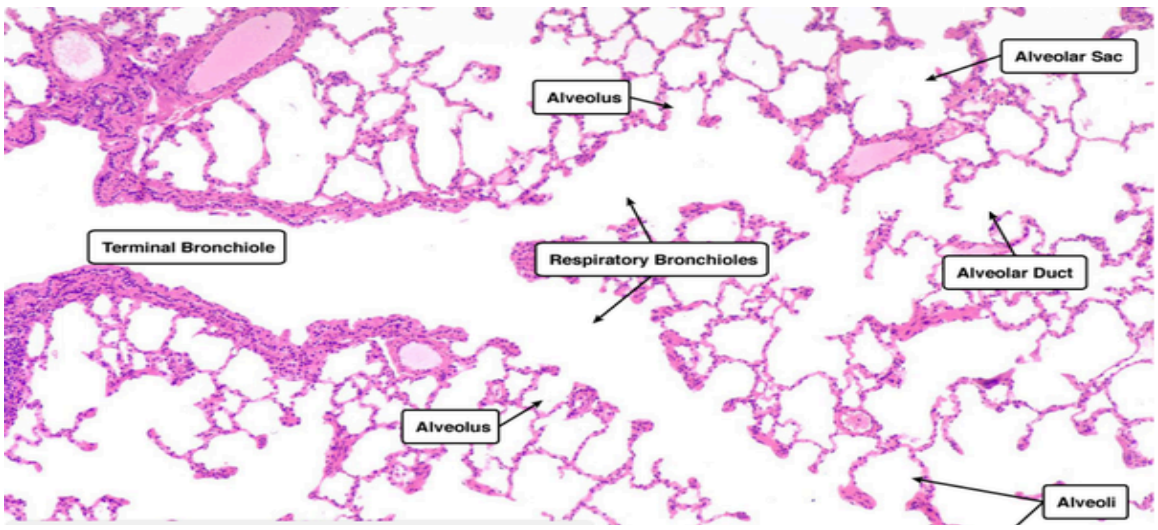


Figure 15. Respiratory portion tissue

Respiratory bronchioles

Its walls are formed by mucosa formed by a cubic epithelium (initially it was cylindrical) with ciliated cells and Club cells (which play a fundamental role in this portion of the respiratory tract) ***Figure 16***. The number of this type of cells will increase progressively.

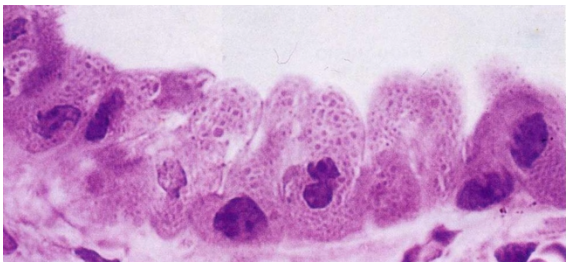


Figure 16. Optical microscope image of Club cells

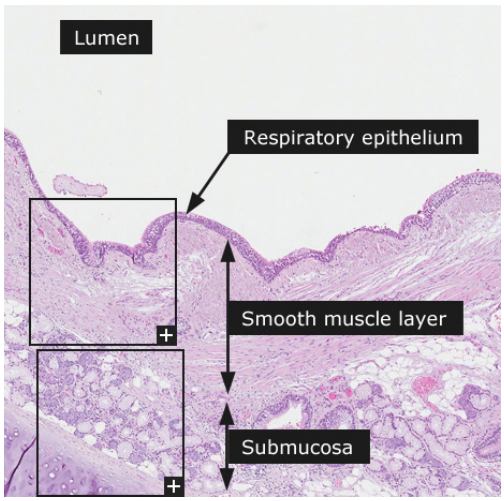


Figure 17. Respiratory bronchioles tissue

The epithelium rests on the basement membrane. Beneath the epithelium is a thin layer of connective tissue called the lamina propria. Neither cartilage nor glands appear; But there is a layer of irregular smooth muscle intertwined with elastic fibers.
Outside there are blood and lymphatic vessels surrounding these bronchioles.

Alveolar duct and alveolar sacs

The alveolar duct has a cylindrical epithelium with ciliated cells and Club cells. There is a basement membrane and a fine connective tissue and underneath smooth muscle, striking since this is the last point where this component appears in the bronchial tree. There are no more 'remains' of bronchiole.
These ducts are continued at the end of the alveolar parenchyma with the alveolar sacs.

The alveolar sac is a saccular-shaped structure full of alveoli (there is no longer a wall). The entrance to the sac can be known as atrium.

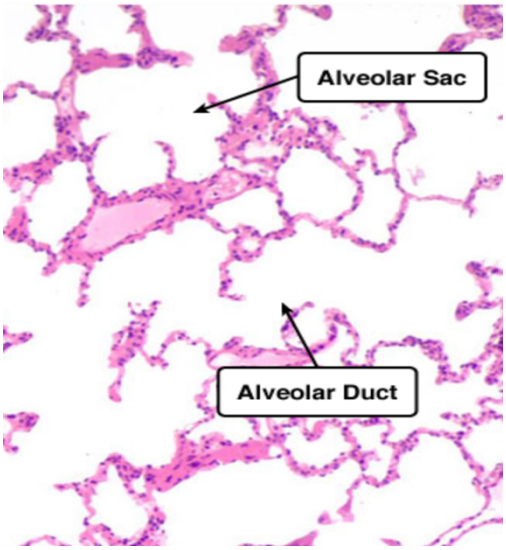


Figure 18. Optical microscope image of the alveolar duct and the alveolar sac

Alveoli

The alveoli are characterized because they are small evaginations found in:

- Respiratory bronchioles
- Alveolar ducts
- Alveolar sacs

The alveolar epithelium is flat and covers the lumen of the alveolus. It is characterized because it is form thanks to the union of Type I pneumocytes and Type II pneumocytes

Type I pneumocyte	Type II pneumocyte
Squamous and extremely thin	Granular and roughly cuboidal
Cover aprox 95% of alveolar surface	Cover aprox 5% of alveolar surface
Involved in gas exchange	Secrete pulmonary surfactant

Table 2. Main characteristics of Type I and Type II pneumocytes

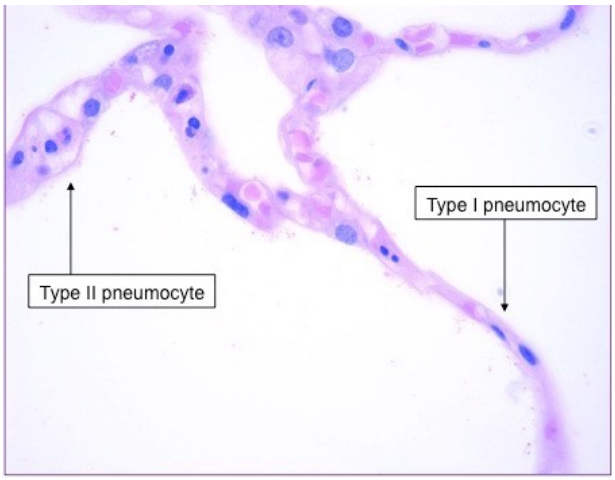


Figure 19. Type I and type II pneumocytes

1.4. Main respiratory diseases

Respiratory disease causes an immense worldwide health burden. It is estimated that 235 million people suffer from asthma, more than 200 million people have chronic obstructive pulmonary disease (COPD), 8.7 million people develop tuberculosis (TB) annually, millions live with pulmonary hypertension and more than 50 million people struggle with occupational lung diseases, totaling more than 1 billion persons suffering from chronic respiratory conditions. At least 2 billion people are exposed to the toxic

effects of biomass fuel consumption, 1 billion are exposed to outdoor air pollution and 1 billion are exposed to tobacco smoke. Consequently, each year 4 million people die prematurely from chronic respiratory disease. It is also important to mention that the most common lethal cancer in the world is lung cancer, which kills more than 1.4 million people each year, and unfortunately the numbers are growing.

No organ is more vital, and no organ is more vulnerable than the lung. Being unable to breathe is one of the most distressing feelings one can have. The lungs are the largest internal organ in the body and the only internal organ that is exposed constantly to the external environment. Everyone who breathes is vulnerable to the infectious and toxic agents in the air. While respiratory disease causes death in all regions of the globe and in all social classes, certain people are more vulnerable to environmental exposures than others. [4]

We will focus on the five more common diseases:

- COPD (Chronic Obstructive Pulmonary Disease): It is characterized by persistent airflow limitation that is usually progressive and that is caused by an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. [5]
- Asthma: When someone have asthma, the airways become swollen. This swelling (inflammation) causes the airways to make thick, sticky secretions called mucus. Asthma also causes the muscles in and around the airways to get very tight or constrict. This swelling, mucus, and tight muscles can make the airways narrower than normal and it becomes very hard for you to get air into and out of your lungs. [6]
- Acute respiratory infections: This infection may interfere with normal breathing. Although it can affect just your upper respiratory system, can also affect the lower respiratory system. It is important to consider that it is particularly dangerous for children, older adults and people with immune system disorders. [7]
- Tuberculosis: TB is caused by slow-growing bacteria called *Mycobacterium tuberculosis*, that when it enters the lungs, they are usually walled off into harmless capsules, causing infection but not disease. Several weeks or month after, these capsules may cause active TB disease. [5]
- Lung cancer: Caused by the malignant growth of cells from the respiratory tract, in particular the lung tissue.

2. *In vitro* methods

First of all, it is important to understand the meaning of *in vitro*.
In vitro methods are methods that are performed with microorganisms, cells or biological molecules outside their normal biological context. Studies conducted using components of an organism that have been isolated from their usual biological surroundings permit a more detailed or more convenient analysis that can be done with whole organisms; however, results obtained may not fully or accurately predict the effects on a whole organism.

2.1. Advantages vs disadvantages

As all biological methods, we have advantages and disadvantages:
On the one hand, *in vitro* studies permit a species-specific, simpler, more convenient and more detailed analysis that cannot be done with the whole organism. On the other hand, the primary disadvantage of *in vitro* experimental studies is that it may be challenging to extrapolate the results back to the biology of the intact organism.

Advantages	Disadvantages
Simplicity: → Living organisms are extremely complex functional systems that are made up of, at a minimum, many tens of thousands of genes, protein molecules, RNA molecules, small organic compounds, inorganic ions, and complexes in an environment that is spatially organized by membranes, and in the case of multicellular organisms, organ systems. **[8]** This complexity makes it difficult to identify the interactions between individual components and to explore their basic biological functions. *In vitro* work simplifies the system under study, so the investigator can focus on a small number of components. **[9]**	**Challenging to extrapolate results→** Results obtained from *in vitro* experiments cannot usually be transposed, as is, to predict the reaction of an entire organism *in vivo*. However, building a consistent and reliable extrapolation procedure from in vitro results to in vivo is therefore extremely important. [10]
Species-specificity→ Human cells can be studied without "extrapolation" from an experimental animal's cellular response.	**Over-interpretation→** Investigators must be careful to avoid over-interpretation of the results, which can lead to erroneous conclusions. [11]
Convenience, automation→ In vitro methods can be miniaturized and automated yielding high-throughput screening methods for testing molecules in pharmacology or toxicology. **[10]**	

Table 3. Main advantages and disadvantages of in vitro methods

As a conclusion, we can say that the main point in which we have to focus when we do *in vitro* experiments is the way that we extrapolate the results to the whole organism, because typically most candidate drugs that are effective *in vitro* prove to be ineffective *in vivo* because issues associated with delivery of the drug to the affected tissues, toxicity towards essential parts of the organism that were not represented in the initial *in vitro* studies, or other issues. [10] [12]

Some solutions could be:

- Increasing the complexity of *in vitro* system to reproduce tissues and interactions between them.
- Using mathematical modeling to numerically simulate the behavior of the complex system, where the *in vitro* data provide provides the parameter values for developing a model.

These two approaches are not incompatible; better *in vitro* systems provide better data to mathematical models. However, increasingly sophisticated *in vitro* experiments collect increasingly numerous, complex, and challenging data to integrate. Mathematical models, such as systems biology models, are much needed here

3. Organs-on-chip

"Organs-on-chips are micro-engineered biomimetic systems containing microfluidic channels lined by living human cells, which replicate key functional units of living organs to reconstitute integrated human organ-level pathophysiology in vitro." These microdevices can be used to test efficacy and toxicity of drugs and chemicals, and to create *in vitro* models of human disease. [13]

Conventional models used in these types of studies, often use living cells cultured in 2D monolayers, 3D extracellular matrix (ECM) gels or multicellular spheroids. Although these *in vitro* models provide controllable environments to probe biological processes at the cellular or tissue level, they lack the ability to replicate organ-specific structural organization or recapitulate integrated physiological functions at the organ level. [13] [14]

Fortunately, recent studies in microengineering technologies, has led to the development of much more sophisticated microfabricated systems commonly known as organs-on-chip which allow one to recapitulate tissue-tissue interfaces, along with complex organ-specific chemical and mechanical microenvironments, to mimic key 3D functional units of living human organs. The main advantage of these microengineering methods is that they enable precise control of feature size on the same scale of living cells or tissues (nanometer to micrometer). [15] [16]

The greater promise of these microsystems approach lies in the possibility of accurately recreating the physical and biochemical microenvironments of the key compartments of living organs that are crucial for reconstituting organ-level functions.

3.1. Lungs-on-chip 1

Microfluidic organ-on-a-chip cell culture technology can be used to create *in vitro* human orthopedic models of small-cell lung cancer (NSCLC) that recapitulate organ microenvironment-specific cancer growth, tumor dormancy, and responses to tyrosine kinase inhibitor (TKI) therapy observed in human patients *in vivo.* [17]

Until now, *in vivo* orthopedic cancer models have been used to improve cancer therapeutics; however, when using these models, it still remains extremely difficult to identify contributions of the microenvironment to tumor growth. Thus, there has been a search for *in vitro* models of human cancer that might provide an alternative approach, models using organ-on-a-chip.

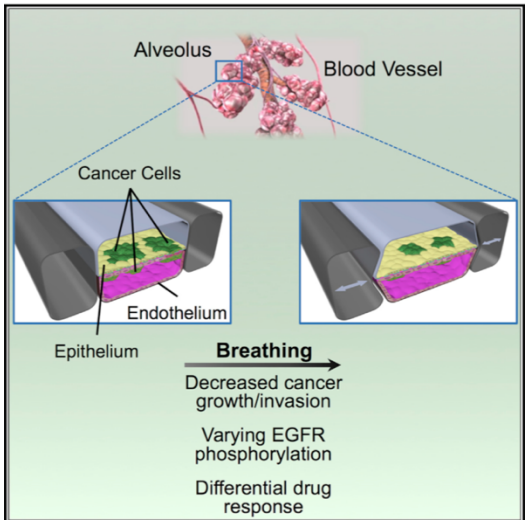


Figure 20: Abstract illustration

As we have said before, organ chips are microfluidic cell culture devices created with microchip manufacturing methods that contain continuously perfused hollow microchannels inhabited by living cells arranged to simulate organ-level pathophysiology. The main advantage of these devices is that they produce levels of tissue and organ functionality not possible with conventional 2D or 3D culture systems. [17]

Results

To create an orthotopic model of human NSCLC representative of the *in vivo* microenvironment, they plated H1975 human NSCLC adenocarcinoma cells at a low density (3,200 cells/cm^2) simultaneously with a 100-fold higher number of primary lung alveolar or small airway epithelial cells on the upper surface of the ECM-coated membrane to integrate the cancer cells into the tissue layers during the tissue differentiation process.

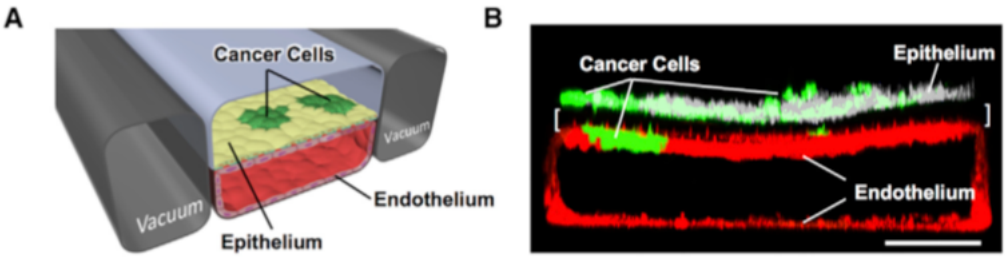


Figure 21: (A) Schematic diagram of a cross-section through the channel microfluidic chip. (B) Confocal fluorescence micrograph of a cross-section of the tow central lined channels of an alveolus chip.

After plating, the medium was removed from the upper channel to create an air-liquid interface (ALI) above the epithelium, while an ALI medium was continuously perfused through the lower vascular channel to feed both tissue layers. As a consequence, the NSCLC cells were engineered to stably express high levels of GFP to optically quantify tumour cell growth and invasion.

When they analyzed the growth of the NSCLC cells that were co-cultured in the human airway chip during the differentiation process, they found that they appeared as isolated GFP-positive cells within the epithelial monolayer after tissue differentiation was completed.

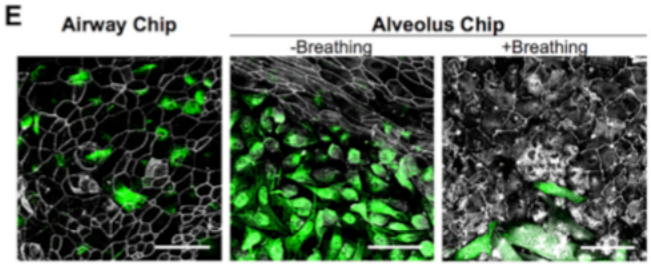


Figure 22. GFP-labeled lung cancer cells (green) exhibit distinct growth patterns within the normal epithelial monolayer

One of the most interesting things is that when they co-cultured the NSCLC in the human alveolus chip, they found that they proliferated much more rapidly than when grown in the airway chip whether cultured in the presence or absence of cyclic mechanical strain to mimic physiological breathing motions. ***Figure 23*** [17]

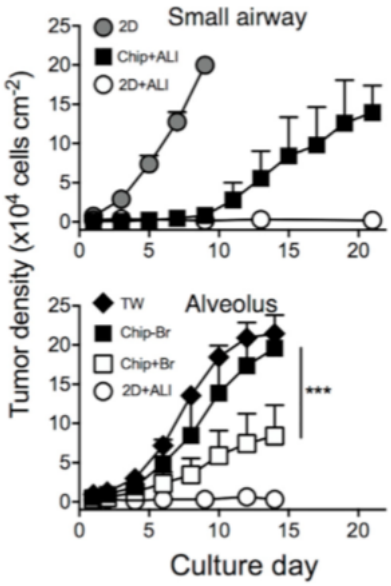


Figure 23. bottom vs top

Modulation of Tyrosine Kinase activity by mechanical strain

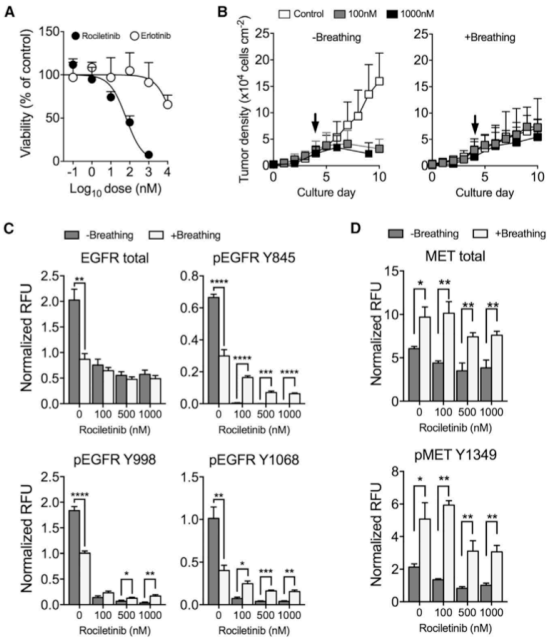
The decreased sensitivity of cancer cells to the TKI drug in the breathing alveolus chip could be due to mechanical regulation of EGFR expression and signaling within the tumor cells, and so we cultured the NSCLC cells with or without cyclic mechanical deformation (10% strain; 0.2 Hz) for 48 hr in a FlexCell culture plate to test this hypothesis independent of other lung cells or fluid flow. Mechanical stimulation produced a significant ($p < 0.05$) decrease in total EGFR protein levels in the NSCLC cells even before rociletinib was added *(**Figure 24C**),* which could possibly account for the observed inhibitory effects of breathing motions on growth and motility. Furthermore, while we found that treatment of these tumor cells with rociletinib resulted in the inhibition of tyrosine kinase phosphorylation at EGFR sites Y845, Y998, and Y1068 in the absence of breathing motions, this inhibition also was greatly diminished in the mechanically stimulated cells (***Figure 24C***). Thus, both downregulation of EGFR and

reduced sup- pression of phosphorylation of the EGFRs that were expressed could explain why the mechanically strained cells were resistant to growth inhibition by this third generation TKI drug (***Figure 24B, right***).

Another important clinical observation is that many patients with the EGFR L858R/T790M mutation who develop resistance to third generation TKIs overexpress the tyrosine protein kinase c-Met, and c-MET overexpression has been implicated as a mechanism of resistance to TKI therapy in NSCLC patients. Consistent with our finding that breathing motions decrease NSCLC cell sensitivity to this TKI, we found that the NSCLC cells significantly increased both their expression and phosphorylation of c-Met when subjected to cyclic mechanical strain, whereas increasing rociletinib dose had no significant effect (***Figure 24D***). Thus, mechanical breathing motions also may suppress NSCLC cell response to TKI therapy by altering this signaling pathway.

Mechanical and therapeutic modulation of Cytokines

Past in vivo studies have revealed that the cytokines, IL-6, IL-8, and VEGF, may serve as clinically important prognostic indicators of cancer growth. Because the alveolus cancer chip model features fluid flow in the vascular channel, we were able to analyze these secreted components within effluents collected over time from this compartment. These studies revealed that IL-6 and VEGF are more abundant, whereas IL-8 was decreased, in the microfluidic alveolar chip model of NSCLC with or without breathing when compared to Transwell cultures without breathing or flow (***Figure 24E***). In addition, treatment of the lung tumor cells with rociletinib significantly reduced levels of IL-6 and IL-8, and increased VEGF levels in both static and breathing alveolus chips; however, the level of suppression of the two interleukins was significantly greater in chips exposed to physiological breathing motions (***Figure 24E***). Importantly, the finding that TKI treatment reduces IL-8 levels in our orthotopic in vitro lung cancer model is also consistent with published results from human clinical trials.



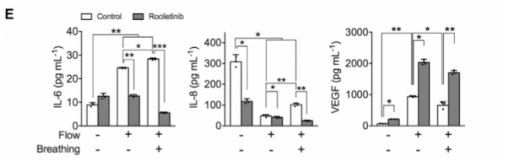


Figure 24: Breathing modulates lung cancer cell responses to a TKI drug.

Discussion

In this study has been shown that the results demonstrate that microfluidic organ chip technology can be used to create *in vitro* human orthotopic lung cancer models that faithfully mimic microenvironment-specific growth patterns as well as permit analysis of molecular level modulation of drug actions by the organ microenvironment. Unfortunately, one important limitation is that this model does not contain tumor stromal cells or immune cells, however these cells can be integrated into these cancer chips in future. Probably, the most important observation is that due to the bottom-up synthetic biology approach, the results indicate that other cell types are not required for expression of the NSCLC phenotypes. In conclusion, the ultimate value of these human orthotopic cancer chip models lie in their potential ability to uncover new insights into mechanisms of cancer control, and to facilitate discovery of novel drug targets and anti-cancer therapeutics. [17]

3.2. Lungs-on-chip 2

Here we describe the development of a human lung 'small airway-on-a-chip' containing a differentiated, mucociliary bronchiolar epithelium and an underlying microvascular endothelium that experiences fluid flow, which allows for analysis of organ-level lung pathophysiology *in vitro*. Exposure of the epithelium to interleukin-13 (il-13) reconstituted the goblet cell hyperplasia, cytokine hypersecretion and decreased ciliary function of asthmatics. Small airway chips lined with epithelial cells from individuals with chronic obstructive pulmonary disease recapitulated features of the disease such as selective cytokine hypersecretion, increased neutrophil recruitment and clinical exacerbation by exposure to viral and bacterial infections. With this robust *in vitro* method for modeling human lung inflammatory disorders, it is possible to detect synergistic effects of lung endothelium and epithelium on cytokine secretion, identify new biomarkers of disease exacerbation and measure responses to anti-inflammatory compounds that inhibit cytokine-induced recruitment of circulating neutrophils under flow. [18]

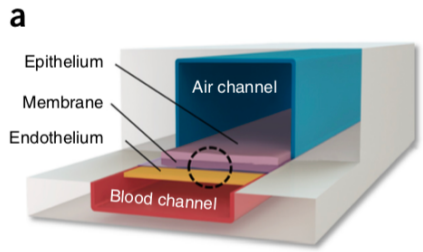


Figure 25: Schematic diagram of a cross-section through the small airway-on-a-chip

To construct the human small airway-on-a-chip the scientist has used soft lithography to create a microfluidic device made of poly(dimethylsiloxane) (PDMS) containing an upper channel separated from a parallel lower microvascular channel by a thin, porous, polyester membrane coated on both sides with type I collagen.

First of all, it was necessary to cultured primary human airway epithelial cells (hAECs) on top of the membrane until confluent with medium flowing in both channels. To trigger the differentiation of lung airway epithelial cells, they removed the apical medium and introduced air to create an air-liquid interface (ALI). They added retinoic acid to the medium flowing in the lower channel to prevent the development of a squamous phenotype. Finally, after several weeks, they seeded primary human lung microvascular endothelial cells on the opposite side of the porous membrane and cultures them at the same flow rate until confluent to create a tissue-tissue interface.

Immunofluorescence confocal microscopic analysis showed that these culture conditions resulted in the formation of a pseudostratified, mucociliary airway epithelium on one side of an extracellular matrix–coated membrane and a planar microvascular endothelium on the opposite side. The epithelial cells were linked by a continuous band of ZO1-containing tight junctions along the lateral borders of their apical surfaces, whereas endothelial cells were joined by adherents' junctions containing PECAM-1. The epithelium formed from cells isolated from the small airway epithelium of lungs from both healthy humans and people with COPD contained many ciliated epithelial cells as well as mucus-producing goblet cells, club cells and basal cells with proportions strikingly similar to those found in normal human lung. Immunodetection of tight junctions was accompanied by the formation of a robust epithelial barrier that restricted the passage of fluorescent inulin (4 kDa) and dextrans (10 and 70 kDa) between the parallel channels during analysis over 24 h, mimicking the barrier function of living lung mucosa. Electron microscopic analysis of the normal lung small airway-on-a-chip confirmed that the cilia on the apical surface of the polarized epithelium had the same structure (9+2 microtubule organization) and length as healthy cilia found in living human lung *in vivo*. High-speed microscopic imaging confirmed that these cilia beat actively at a frequency of 9–20 Hz, which again is nearly the same as that observed in human airways (***Figure 26***). Moreover, when we measured mucociliary transport by introducing fluorescent polystyrene microbeads into the top channel, we observed rapid coordinated movement of the beads along the surface of the epithelium as a result of active synchronized cilia beating; again, the particle velocity measured was nearly identical to that observed in healthy human lung airway (***Figure 26***). Thus, the human lung small air- way-on-a-chip effectively recapitulated many of the structures and functions of healthy lung bronchioles and sustained them for weeks *in vitro*. [18]

	Human airway	Small airway-on-a-chip
Cell type		
Ciliated cells (%)	~30	~20–30
Goblet cells (%)	~10–15	~10–20
Club cells (%)	~11–44	~25
Basal cells (%)	~6–30	~20
Cilia		
Structure	9 + 2 structure	9 + 2 structure
Length	~6 μm	~6 μm
Beating frequency	9–20 Hz	9–20 Hz
Transport velocity	40–150 μm s^{-1}	40–100 μm s^{-1}

Figure 26: Comparison of structure and function between living human airway and human small airway-on-a-chip

Discussion

In conclusion, we can say that the small airway-on-a-chip described achieved grater robustness and fidelity in mimicking the structure and functions of the human lung airways, as well as in modelling pulmonary diseases and recapitulation of lung inflammatory responses in vitro.

As a consequence of using organs on-a-chip, the main advantage is that it is possible to vary virtually all systems parameters while simultaneously analysing human organ-level responses in real time with molecular-scale resolution.

3.3. Lungs-on-chip 3

Here, we describe a biomimetic microsystem that reconstitutes the critical functional alveolar-capillary interface of the human lung. This bioinspired microdevice reproduces complex integrated organ-level responses to bacteria and inflammatory cytokines introduced into the alveolar space. In nanotoxicology studies, this lung mimic revealed that cyclic mechanical strain accentuates toxic and inflammatory responses of the lung to silica nanoparticles. Mechanical strain also enhances epithelial and endothelial uptake of nanoparticulates and stimulates their transport into the underlying microvascular channel. Similar effects of physiological breathing on nanoparticle absorption are observed in whole mouse lung. Mechanically active “organ-on-a-chip” microdevices that reconstitute tissue-tissue interfaces critical to organ function may therefore expand the capabilities of cell culture models and provide low-cost alternatives to animal and clinical studies for drug screening and toxicology applications. [19]

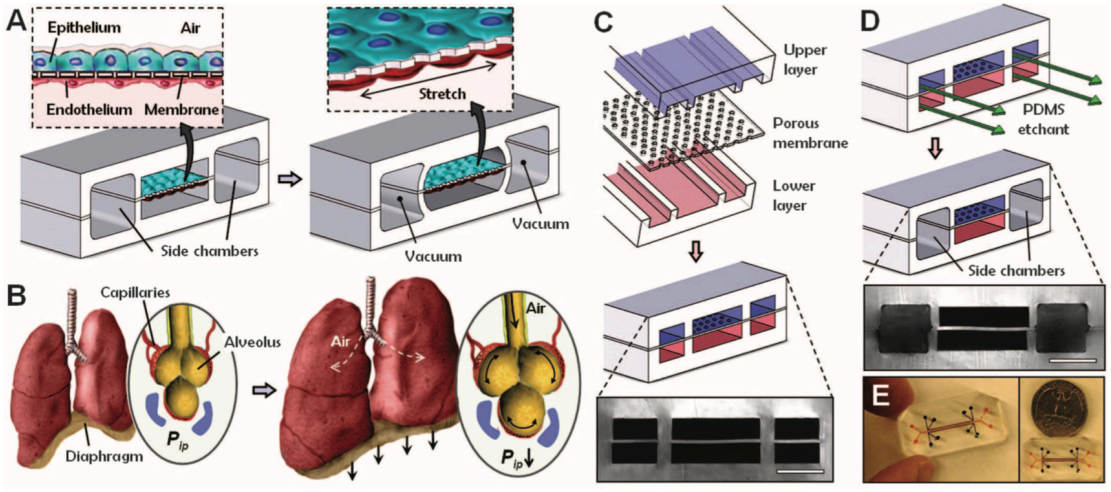


Figure 27: Biologically inspired design of a human breathing lung-on-a-chip microdevice

(A)The microfabricated lung mimic device uses compartmentalized PDMS microchannels to form an alveolar-capillary barrier on a thin, porous, flexible PDMS membrane coated with ECM. The device recreates physiological breathing movements by applying vacuum to the side chambers and causing mechanical stretching of the PDMS membrane forming the alveolar-capillary barrier. ***(B)*** During inhalation in the living lung, contraction of the diaphragm causes a reduction in intrapleural pressure (P_{ip}), leading to distension of the alveoli and physical stretching of the alveolar-capillary interface. ***(C)*** Three PDMS layers are aligned and irreversibly bonded to form two sets of three parallel microchannels separated by a 10-mm-thick PDMS membrane containing an array of through-holes with an effective diameter of 10 mm. ***(D)*** After permanent bonding, PDMS etchant is flowed through the side channels. Selective etching of the membrane layers in these channels produces two large side chambers to which vacuum is applied to cause mechanical stretching. ***(E)*** Images of an actual lung- on-a-chip microfluidic device viewed from above. [19]

Discussion

If we talk bioethically, biomimetic microsystems are the perfect replacements for animal testing. Development of cell-based biochips that reproduce complex, integrated organ-level physiological and pathological responses could revolutionize many fields, including toxicology and development of pharmaceuticals and cosmetics that rely on animal testing and clinical trials as we have said several times.

This human breathing lung-on- a-chip microdevice provides a proof of principle for this novel biomimetic strategy that is inspired by the integrated chemical, biological, and mechanical structures and functions of the living lung. This versatile system enables direct visualization and quantitative analysis of diverse biological processes of the intact lung organ in ways that have not been possible in traditional cell culture or animal models.

There are still differences between our lung mimic device and the alveolar-capillary barrier in vivo (e.g., barrier thickness, cellular composition, lack of alveolar macrophages, and changes in air pressure and flow), and the transformed lung cells used in our study might not fully reproduce the responses of native alveolar epithelial cells. However, our data clearly demonstrate that this biomimetic microsystem can reconstitute multiple physiological functions observed in the whole breathing lung.

Microengineering approaches developed in this research also might offer new opportunities to more accurately model critical tissue-tissue interfaces and specialized physical microenvironments found in other organs, such as the gut, kidney, skin, and bone marrow, as well as in human cancers. These biomimetic microsystems are miniaturized and can be easily multiplexed and automated. Hence, with simplified designs and careful choice of biocompatible device materials, they might be useful for high-throughput analysis and screening of cellular responses to drugs, chemicals, particulates, toxins, pathogens, or other environment stimuli relevant to pharmaceutical, cosmetic, and environmental applications. Furthermore, these microengineering approaches might open the possibility of integrating multiple miniaturized organ model systems into a single device to recapitulate interactions between different organs and enable more realistic in vitro assays of the whole body's response to drugs and toxin. [20] [21]

4. Main elements for the design

Here we describe the main elements for the fabrication, microengineering and operation of these microfluidic organ-on-chip systems.

The first point to bear in mind is how a biomimetic 3D microfluidic system for the coculture of human alveolar epithelial cells and pulmonary microvascular endothelial cells to create the human 'breathing' lung-on-a-chip is made.

Organs in the human body are complex living systems composed of different types of tissues that form complex tissue-tissue interfaces, such as between endothelium-lined blood vessels and parenchymal epithelial cells that exhibit organ-specific functions. Most organs are multi-modular structures in that they consist of repeating smaller functional units that individually perform the major characteristic functions of the whole organ (e.g., gas exchange in the alveoli of the lung). Typically, these functional units comprise different types of specialized tissues (e.g., epithelium, vascular endothelium, connective tissue, immune cells, nerves, etc.) that interface in organ-specific patterns and are subjected to dynamic changes in chemical and mechanical signals that vary depending on their particular spatial microenvironment.

To develop a useful organ surrogate device for *in vitro* analysis of complex human physiology, it is necessary to both reproduce normal tissue-tissue interfaces and mimic this complex physical microenvironment in which cells are normally situated. Scientists recreated the critical alveolar-capillary interface of the lung air sac in our human breathing lung-on-a-chip [19] by developing a 3D microfluidic device that contains two parallel microchannels with the same dimensions (400 μm wide × 100 μm high) separated by a thin porous flexible membrane made of poly(dimethylsiloxane) (PDMS) ECM-coated. We chose the width of microchannels to approximate the average diameter of an alveolus in human lungs. These channels also are surrounded on either side by two hollow full-height chambers that permit application of cyclic suction to mechanically stretch and relax the flexible PDMS membrane in the central channel. This design was inspired by the mechanism of physiological breathing in living human lungs in which sub-atmospheric pressure in the intrapleural space generated by inspiration causes the inflation of the alveolar air sacs, which induces filling of the lungs with air and stretching of the alveolar epithelium and the juxtaposed vascular endothelium in the surrounding capillaries. [19] Human alveolar epithelial cells and pulmonary microvascular endothelial cells are then introduced into the upper and lower microchannels, respectively, and grown on the ECM-coated membrane to form two closely apposed tissue monolayers.

During cell culture, medium is perfused continuously through both channels to provide cells with nutrients and to remove their metabolic wastes. Once confluence is reached, the culture medium is removed from the upper channel, and this channel is filled with air to form an air-liquid interface at the apical surface of the alveolar epithelium, which induces the cells to differentiate and express tissue-specific functions, such as surfactant production. During this period, medium flow is maintained in the lower channel to recapitulate the dynamic flow that normally perfuses the pulmonary capillary blood

vessels and supplies oxygen and nutrients to the epithelium through the intervening endothelium.

To mimic physiological breathing motions of the lung, vacuum is applied to the side hollow chambers to induce outward bending of the elastic vertical walls of the central microchannels. This deformation laterally stretches the intervening PDMS membrane along with the attached epithelial and endothelial monolayers. When the vacuum is removed, the elastic recoil of PDMS causes the membrane to return to its original length, thus relaxing the tissue layers. By repeating this actuation cycle at a frequency of 0.25 Hz and controlling the level of stretching to produce ~10% cyclic strain, it becomes possible to generate dynamic mechanical distortions similar to those observed in the air sac of the living human lung. [13]

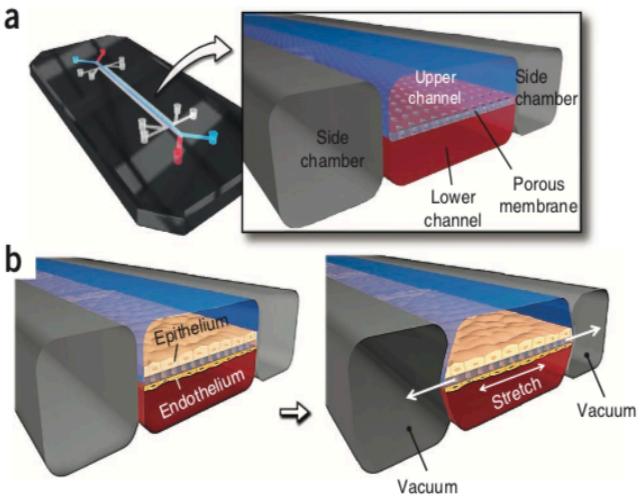


Figure 28. Mechanically active organ-on-chip microdevice with compartmentalized 3D microarchitecture

(a) The human lung-on-a-chip microsystem is constructed in a multilayered microfluidic device comprising the upper (blue) and lower (red) cell culture microchannels with a microfabricated porous elastic membrane sandwiched in-between. The microdevice is also equipped with two full-height, hollow microchambers alongside of the cell culture channels.

(**b**) Physiological breathing motions in the living human lung are reproduced by the application of vacuum to the side chambers. This actuation causes the lateral elongation of the intervening elastic membrane, which induces mechanical stretching of the adherent tissue layers in the central channels.

5. Working in laboratory

Once we have analysed the main elements for the design of a lung-on-chip, we are going to detail a protocol that provides a step-by-step procedure for creating 3D organ-on-chip microfluidic system that enable one to form one or two closely apposed confluent monolayers of differentiated human cells and to expose them to physiologically relevant mechanical and biochemical cues in polarized microenvironments. [13]

1. **Fabrication of SU-8 silicon master and its silanization**

   SU-8 is a high contrast, epoxy-based photoresist designed for micromachining and other electronic applications where a thick chemically and thermally stable image is desired.

2. **Creation of the upper and lower microchannels**

   The fabrication of the upper and lower microchannels take place in a very similar way. We are going to explain how to create the upper microchannels:

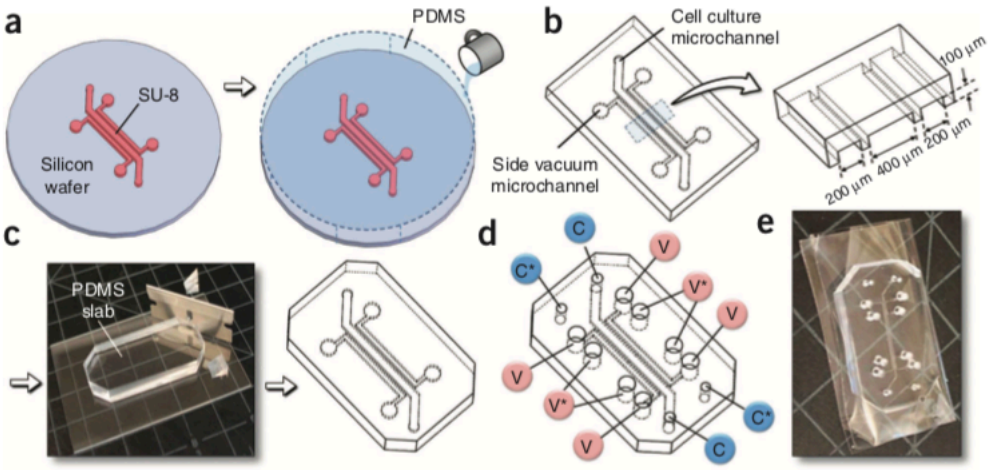


Figure 29. Fabrication of the upper microchannels of the lung-on-a-chip

(**a**) Prepolymer of PDMS mixed with curing agent is poured onto the photolithographically prepared SU-8 microchannel features and subsequently cured at an elevated temperature. (**b**) The fully cured PDMS is peeled off of the master and cut into a rectangular block embossed with three parallel upper microchannels that are 100 µm in thickness. The widths of the central cell culture channel and two side vacuum channels are 400 µm and 200 µm, respectively. (**c**, **d**) The corners of the PDMS slab are removed (**c**), and access ports are made to the microchannels using hole punchers (**d**). Cell culture and vacuum channels are denoted by C and V, respectively, and asterisks mark access holes for the lower microchannels. (**e**) The upper PDMS slab is cleaned and wrapped in packaging tape for later use.

3. Fabrication of porous PDMS membranes of the lung-on-a-chip

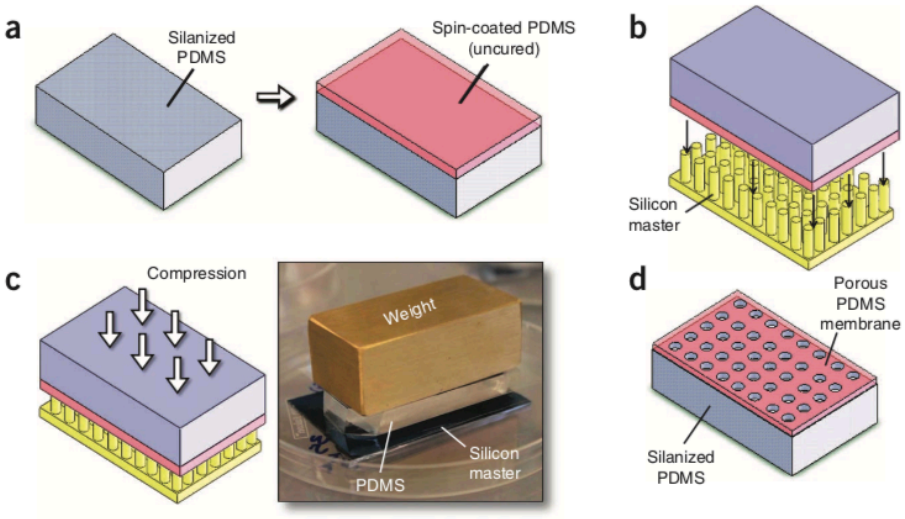


Figure 30. Fabrication of porous PDMS membranes

(**a**) PDMS is spin-coated on a silanized PDMS slab to form a 10-µm-thick film of uncured PDMS. (**b**, **c**) Subsequently, the PDMS slab is placed on a silicon wafer patterned with an array of microfabricated pillars (**b**) and compressed uniformly against the master using weight during PDMS curing (**c**). The diameter and height of the pillars are 10 µm and 50 µm, respectively. (**d**) After complete curing of PDMS, the weight and silicon master are removed to produce a 10-µm-thick PDMS membrane with microfabricated through-holes that is reversibly attached to a silanized PDMS surface.

4. Alignment, assembly and chemical etching of PDMS membrane of the lung-on-a-chip

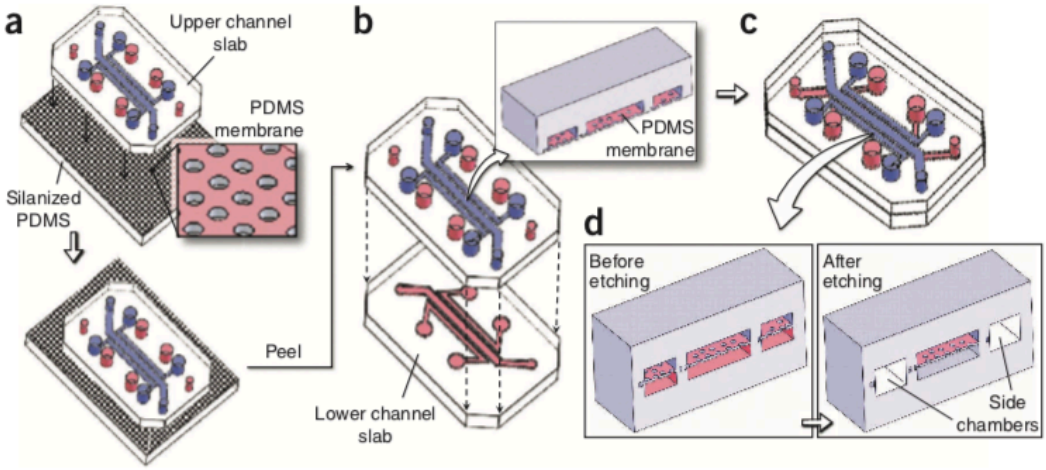


Figure 31. Alignment, bonding and chemical etching of the lung-on-a-chip microdevice

(a) After a brief surface treatment with corona, the upper PDMS slab is irreversibly bonded to the membrane. **(b)** Once bonding is accomplished, the upper microchannel slab carefully separated from the silanized PDMS block, primed with corona and aligned/bonded to the lower microchannel. **(c, d)** This results in the production of a fully assembled microfluidic device **(c)** in which the membrane layers in the vacuum microchannels are etched away to form two hollow side chambers **(d).**

5. **Incorporation of fluidic interconnects and sterilization of the lung-on-a-chip**

   The fully assembled device is placed into a UV-ozone cleaner, where it is sterilized. This process, eliminates, removes, kills or deactivates all forms of live (in particular microorganisms) and other biological agents present in the surface.

6. **Microfluidic cell culture in the lung-on-a-chip**

   ECM coating solution is introduced into both the upper and lower microfluidic channels. Alveolar epithelial cells and pulmonary microvascular endothelial cells are seeded. (Annex)

7. **Analysis of microengineered tissues**

   When the microfluidic cell culture is complete is necessary to:

   A) Assess the viability of the cultured cells.
   B) Assess barrier integrity via immunofluorescence staining of intracellular junctions.
   C) Measure trans-bilayer electrical resistance

To resume the steps to follow in laboratory we have the following table:

Steps	Main objectives	Common failures	Timing
1	Master fabrication	Possible formation of air bubbles in the poured photoresist.	17 h
2	Fabrication of the upper and lower microchannels	PDMS channel slabs with uneven surfaces. Using large air pressure causing unwanted detachment of SU-8 microchannel features from a silicon wafer.	6,5 h
3	Membrane fabrication	Blockage of membrane pores. Membrane rupture.	1,4 d

4	Device alignment and assembly	Misalignment of microchannel walls. Rupture of PDMS membranes during multiple attempts to align the microchannels.	1,1 d
5	Chemical etching	Leakage of chemical etchant into the cell culture channels due to hydrophobicity of PDMS channel surfaces.	20 min
6	Installation of interconnects and device sterilization	Non uniform chemical etching generates variations in the thickness of the vertical walls separating the central cell culture channels from the side vacuum chambers, leading to varying degrees of membrane stretching along the channel length. Forget to sterilize immediately before use.	1,5 h
7	Microfluidic cell culture	Lack of cell attachment. Cell death and detachment caused by the detrimental mechanical forces generated by air bubbles formed in the cell culture channels as a result of evaporation. Unwanted cell injury and death during the withdrawal of culture medium from the upper channel to form an air-liquid interface.	5-21 d
8	Analysis of microengineered tissues		30 min-1.25 d

Table 4. Steps, main objectives, common failures and timing in laboratory

The total time required to fabricate these microdevices can potentially be substantially reduced by baking PDMS at a higher temperature (e.g., 85 °C) to shorten the curing process from 4–5 h to 1 h for the production of the upper and lower microchannel slabs and porous membranes. This also can eliminate the need for overnight curing after corona treatment to achieve irreversible bonding between PDMS layers and allow for completion of the bonding process within a few hours. Under these conditions, the total fabrication time can be reduced from 3.5 d to 2.1 d. [13]

We are going to show an example of a lung-on-a-chip microfluidic device produce by the fabrication protocol just described.

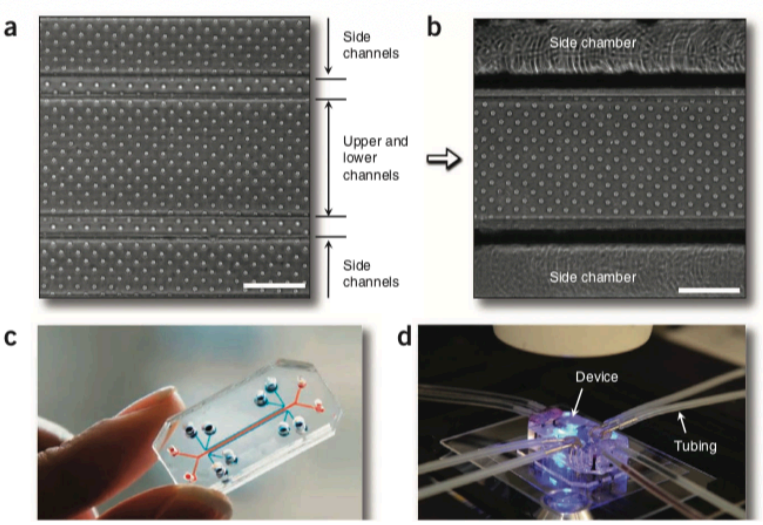


Figure 32. A multi-layered 3D microfluidic device for the production of the human breathing lung-on-a-chip

(a) A micrograph of well-aligned upper and lower microchannels separated by a thin porous PDMS membrane in an assembled microdevice. (**b**) Chemical etching of PDMS successfully removes the porous membrane in the side channels and causes the thinning of the PDMS walls between the central microchannels and the side chambers. (**c**) Optical transparency of PDMS permits direct visualization of individual microchannels embedded in the device. In this image, the cell culture microchannels and vacuum chambers are shown in orange and blue, respectively. (**d**) Tubing is attached to the assembled device by using bent needles inserted into the access ports. The final PDMS microdevice can be placed on a regular microscope stage for imaging.

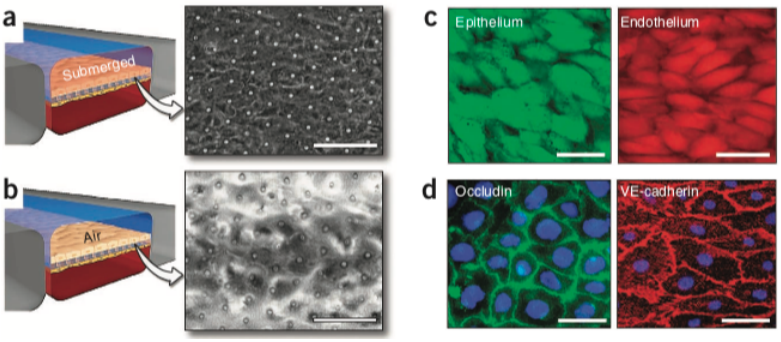


Figure 33. Production and microfluidic engineering of the alveolar epithelium and microvascular endothelium in the lung-on-a-chip microdevice

Human lung epithelial cells and pulmonary microvascular endothelial cells seeded into this device proliferate continuously over the period of 5–7 days to cover the entire surface of the membrane on their respective side **(a)**. Successful formation of these confluent monolayers prevents leakage of culture medium from the lower microchannel into the upper chamber during air-liquid interface culture **(b)**. If the epithelial cells are prematurely exposed to air before they are allowed to form a confluent monolayer, the upper alveolar channel can become flooded with culture medium infiltrating from the lower channel; in this case, cultures need to be maintained longer under flow in both channels before air is introduced into the upper channel **(c).** 10% linear cyclic strain is applied to the alveolar-capillary barrier for 3 days before the end of air-liquid interface culture to mimic physiological breathing, and this does not compromise barrier integrity. At the termination of air-liquid interface culture, the epithelial and endothelial cells remain viable and form intact homogeneous cell monolayers that express high levels of intercellular junctional proteins, such as occludin and VE-cadherin **(d).**

5.1. Annex: Cell seeding protocol [13]

Timing: 5-21 days

1) Introduce 500 μl ECM coating solution into both the upper and lower microfluidic channels and pinch off tubing to prevent coating solution from leaking out of the device. It is important to ensure that the entire microchannels are filled with the ECM solution without any trapped air bubbles.
2) Place the microdevice in a humidified 37 °C incubator overnight
3) Flush the channels with medium to remove residual coating solution and return the microdevice to the incubator for 2 h.
4) Remove the culture medium from a T75 flask containing 80–90% confluent human alveolar epithelial cells. Wash the cells with Ca^{2+}-free and Mg^{2+}-free PBS twice.
5) Add 1 ml of prewarmed trypsin/EDTA solution (0.05%) to the cell culture flask and incubate the mixture in a humidified incubator (37 °C, 5% CO_2) for 5–7 min or until the cells are released from the growth surface. Count and pellet 250,000 cells by centrifugation at 220*g* for 5 min.
6) Thoroughly resuspend the epithelial cell pellet in 50 μl of serum-containing epithelial culture medium and load it into a 1-ml syringe.
7) Use clamps to close the inlet and outlet of the lower microchannels and introduce the concentrated cell suspension into the epithelial compartment through the outlet of the upper microchannel.
8) Clamp closed the inlet and outlet ports of the upper microchannel to allow the seeded epithelial cells to attach to the membrane surface.
9) Incubate the device at 37 °C for 3–4 h.
10) Inspect the microchannels under a microscope to ensure cell adhesion to the membrane.
11) Connect the device to a syringe pump and perfuse both central microchannels with epithelial culture medium at a volumetric flow rate of 20 μl h^{-1} in a humidified incubator maintained at 37 °C.
12) On the following day, repeat Steps 4–6 to harvest and resuspend primary human pulmonary microvascular endothelial cells.
(Before seeding of endothelial cells, ensure that the majority of the pores on the PDMS membrane are covered by the attached epithelial cells to prevent inter-compartment cell migration through the membrane pores during culture.)
13) Disconnect the device from a syringe pump and close the upper microchannel.
14) Invert the microdevice and slowly inject the endothelial cell suspension into the outlet port of the lower channel by using the same method described in Step 7.
15) Pinch off tubing connected to the inlet and outlet of the lower channel, and incubate the device, inverted, in a humidified 37 °C incubator for 3–4 h.
16) Once endothelial cell attachment is accomplished, reconnect the device to the pump and perfuse the upper and lower channels with epithelial and endothelial culture medium, respectively, at a volumetric flow rate of 20 μl h^{-1} in a humidified incubator maintained at 37 °C.

17) Allow the cells to grow to confluence over the course of 5–7 d. Add dexamethasone to the epithelial cell culture medium on day 3. (Dexamethasone strengthens the structural integrity of the alveolar epithelial monolayer.)
18) Once cells form a confluent monolayer, gently aspirate the epithelial cell culture medium from the upper channel to create an air-liquid interface over the apical surface of the alveolar epithelial cells.
19) Replace the culture medium in the lower channel with a 50/50 mixture of epithelial and endothelial media containing 1 μM dexamethasone. Continue microfluidic cell culture in this manner for 15 d.
20) On day 15, apply cyclic strain to the cell populations via the side chambers by using a vacuum pump controlled by the Flexcell Tension System. Apply cyclic strain of 10% at a frequency of 0.2 Hz in a sinusoidal waveform to mimic physiological breathing in the human lung.

6. Conclusions

Unfortunately, animal testing has been until now the main method for simulating and predicting human responses to drugs, chemicals, pathogens and environmental toxins; however, animal studies are costly, lengthy and controversial, and their results often fail to predict human responses. These problems raise serious economical, ethical and scientific issues principally in biomedical studies. Therefore, it was in dire need to find alternatives to these conventional methods. The greater promise was these biomimetic microsystems called Organs-on-chips, which represents a low-cost solution and has the possibility of recreating the physical and biochemical microenvironments of the key compartments of living organs that are crucial for reconstituting organs-level-functions.

The main challenge for the field has been to develop microsystems that reconstitute complex functionality of living organs by fully integrating multiple tissues, recreating crucial tissue-tissue interfaces characteristics of each organ's specific 3D microarchitecture and reconstituting their physiological mechanical and biomechanical microenvironments. Nevertheless, studies are revolutionizing many fields, specifically biomedical engineering.

As we have centered our study in the lung-on-a-chip, researchers have shown that these microdevices, provides unprecedented capabilities to reconstitute, directly observe and quantitatively analyze organ-level physiological functions such as gas exchange and immune responses to bacteria, nanoparticulates, etc. Although there are still differences between our lung and these microdevices, it represents an innovative screening platform that could potentially replace *in vivo* assays in the near future or substantially improve the outcome of animal and clinical studies, by enhancing the predictive power of *in vitro* or in silico computational models.

In conclusion, not only the main objective of researchers around the world is to manufacture miniaturized microsystem that can be easily multiplexed and automated, with simplified designs and biocompatible device materials, but also open the possibility of integrating these individual organs-on-chip models in a single instrument to recapitulate multiorgan interactions and whole-body physiology.

7. Future lines

Lab-on-a-chip technology has become reality recently, where experts from computer industry by fabricating microprocessors applied to the miniaturization of biological systems, have revolutionized the scientific landscape.

We have reviewed recent progress in organs-on-a-chip technology, specifically lungs-on-chip. The development of these microdevices, has attracted world-wide research attention and great scientific advances have been made in the biomedical engineering field, indeed.

Prior to 2012, the biomedical applications of organs-on-a-chip resided mainly within academic research laboratories and much innovative work continues there. However, over the past decade, the devices have become more robust and currently several pharmaceutical companies are trialing devices. Although organs-on-a-chip technology has developed rapidly, the human-on-a-chip theory remains distant.

As we have described before, PDMS is the most widely employed material, but comes with disadvantages as the resultant film is thicker that the *in vivo* morphology. So, the first point to bear in mind is that researchers have to step forward in order to identify suitable alternative biomaterials. Moreover, the cost of manufacturing and experimental implementation is relatively expensive, which is not conducive to the widespread use of organ chips, therefore we have to look components of low cost and easy to dispose.

In terms of integrated system components, the media volume and connector size must be reduced for general use. Collecting samples on the chip may interfere with its operation, resulting in changes in the concentration of various metabolites. Therefore, more suitable sensors are required. Universal cell culture mediums suitable for all organs are also required.

To conclude, looking to the future, the main goal will be to integrate numerous organs into a single chip, and to build a more complex multi-organ chip model, always having in mind that as the number of organs increases, functionality becomes more complex and it will generate more data that has to be process correctly. After that, we will finally achieve our “Human-on-a-chip”.

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