Design and development of a novel Crystal ribcage for probing real-time lung function at cellular resolution in health and disease

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Method Article

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Abstract

Multiple diseases impact the mechanical, biological, and immune function of the lung at the cellular scale. Despite recent progress in intravital imaging, optical imaging of the lung during active respiration and circulation has remained challenging. We introduce here the crystal ribcage: a transparent ribcage that (i) allows truly multiscale optical imaging of the lung in health and disease from whole-organ to single cell, (ii) enables modulating the lung biophysics and immunity through intravascular, intrapulmonary, intraparenchymal, and optogenetic interventions, and (iii) preserves the 3-D architecture, air-liquid interface, cellular diversity, and respiratory-circulatory functions of the lung. We probed how diseases like cancer and pneumonia progression remodels the respiratory-circulatory functions at the single alveolus and capillary levels. The crystal ribcage and its broad applications will facilitate further studies of real-time remodeling of the alveoli and capillaries during pathogenesis of nearly any pulmonary disease, leading to the identification of new targets for treatment strategies.

Introduction

The lung is continuously exposed to mechanical, biological, and immunological stresses throughout its lifetime, and it is the site of many fatal pathologies such as primary and metastatic cancers, respiratory infections, and both obstructive and fibrotic diseases among many others. Exemplified by the COVID-19 pandemic, our understanding of lung pathophysiology is limited. Continuous mechanical motion and enclosure by the ribcage makes the lung one of the most challenging organs to study at cellular resolution in real-time keeping the key physiological functions intact. Clinical imaging modalities (e.g., MRI, CT, and PET) lack the spatiotemporal resolution needed to resolve dynamic alveolar respiratory function, capillary flow, and cellular trafficking and motility. Histological analysis can visualize fixed lungs at subcellular resolution, compromising the temporal dynamics of lung micro-physiology which is entirely lost in these “snapshot” images. Recent advances in intravital microscopy has enabled high resolution imaging of microcirculation in real-time\(^1\)-\(^5\), which led to major discoveries in lung biology and immunity\(^2,5\)-\(^7\). However, these methods compromise the respiratory function of the lung, a defining feature of the pulmonary system with crucial effects on the physics, biology, and immunity of the lung\(^8\)-\(^11\), and is limited to visualizing only a few alveoli in a specific anatomical location of the lung through a small, fixed imaging window.

Here, in a major departure from existing approaches, we introduce the crystal ribcage, a transparent ribcage that enables (a) optical imaging of nearly the entire surface of a functioning murine lung, and (b) real-time probing of the lung at cellular resolution while its respiratory functions are preserved via positive- or negative-pressure ventilation (as in mechanically assisted or spontaneous breathing), and its circulatory functions are preserved through ex vivo pulmonary perfusion of whole blood or media of interest. The crystal ribcage combines the in vivo benefits, i.e., preserving the cellular diversity and complex architecture of the lung, with the benefits of organ-on-chip models, i.e., imaging capability and high controllability of microphysiology. These unique capabilities of the crystal ribcage in perturbing the
biophysics and immunity of the lung through intrapulmonary, intravascular, and optogenetic interventions while visualizing real-time cellular events in a functioning lung provide enormous opportunities for understanding cause-effect relationships in the pathogenesis of pulmonary diseases.

We designed the crystal ribcage to mimic the geometry and key properties of the native ribcage to preserve the in vivo physiological conditions of the mouse lung during imaging. We successfully imaged the whole distal lung surface as a whole lobe or the necessary anatomical location in its native geometry using a bottom-up laser scanning confocal and top-down multi-photon microscope as well as a top-down optical coherence tomography probe and capture spatial heterogeneities from the lung apex down to the base on both the right and left sides of the lung. We demonstrated the versatility of crystal ribcage to probe lung dysfunction in nearly any pulmonary disease or condition with parenchymal presentation, such as primary and metastatic breast cancer, pneumonia, emphysema (or chronic obstructive pulmonary disease), and fibrosis at the whole organ, whole lobe, and alveolar scales. We focused our primary study using the crystal ribcage on models of cancer to visualize the impact of tumors on the function of alveoli and the remodeling of the peri-tumor microenvironment, the immune response to bacterial infection and acute injury, and we also briefly studied mouse models of pulmonary fibrosis and emphysema at multiple scales from whole lobe down to single cells in capillaries in real-time, which was not possible using previous imaging approaches. Finally, the unique capabilities of crystal ribcage in imaging and controllability of a functioning lung are scalable to human whole-lung, and adaptable to other organs such as heart, brain, and liver. Given the recent advances in long-term maintenance of large animal and human lung, brain, and liver, our transformative approach opens new avenues in transplantation and organ regeneration research in addition to understanding the real-time cellular pathogenesis in functional organs.

Reagents

1. Formlabs Clear or GreyPro resin (Formlabs, MA, USA)
2. KRAZY glue (Newell Office Brands, GA, USA)
3. Ecoflex 00-30 (Smooth-On Inc, Macungie PA)
4. Polymethyldissiloxane (Sylgard 184 Silicone Elastomer Kit, Dow Chemicals, MI, USA)
5. PDMS-PEG Copolymer (DBE-712, Gelest Inc., Morrisville, PA)
6. Casting sugar (Isomalt, Fancy Sweets LLC, IL, USA)
7. Polystyrene sheets (MEGA FORMAT Plastics, NJ, USA)
8. 70% Isopropyl alcohol (IPA)

Equipment
1. Formlabs Form3 Resin 3D printer (Formlabs, MA, USA)

2. Dental vacuum forming machine (JT-018, Yae First Trading Co., China)

3. Round le (1/8”) (4233A11, McMaster-Carr, NJ, USA)

4. Round le (5/32”) (4233A17, McMaster-Carr, NJ, USA)

5. Dehydrating oven (31190C, Hamilton Beach Brands, VA, USA)

6. Harrick Plasma Cleaner (Harrick Plasma, NY, USA)

**Procedure**

**A. Mouse ribcage 3-D object creation**

1. Segment the mouse chest cavity from previously recorded pressure controlled µCT scans to obtain an 3D STL file of the chest cavity. We collaborated with the Hoffman group at the University of Iowa to get C57B/6 and AJ mice chest cavity scans, and the Boston University Micro-CT imaging facility helped us get FVB mouse chest scans. We segmented the images in MATLAB (can be performed in any software of choosing, eg. Slicer3D, Blender, Python) to obtain a coarse 3D object that represented the mouse chest cavity including a small section of the trachea.

2. The segmented 3D STL was imported into Solidworks to add registration features prior to 3D printing. The model could also be scaled in SolidWorks to be age-specific by scaling the binary object based on previously reported lung volume-age studies.

3. The final STL file was printed using FormLabs clear or grey pro resin on a Form3 printer. The 3D printed object was washed in IPA for 20 minutes and cured under UV at 60°C for 45 minutes. The final 3D printed can be further washed and polished to remove any layer lines and obtain a high gloss finish on the surface.

**B. Deformable PDMS ribcage fabrication**

1. The 3D printed lung insert was used to make a negative mold by embedding it in Ecoflex 00-30, a soft silicone-based elastomer called under vacuum for 5 hours.

2. Post curing of the Ecoflex the 3D insert was removed to obtain a smooth exact negative mold.
3. The internal surface of the mold was coated with a thin layer of 10:1 PDMS to completely seal it from air and make the internal geometry smooth. The coated ecoflex mold was cured at ~110°C for 1 hour. The mold can be reused for multiple ribcages now.

4. The negative space was slowly filled with a casting sugar solution that was kept liquid at ~140°C. The sugar filled mold was degassed in an oven for 1 hour to remove any trapped air bubbles in the sugar.

5. The sugar mold was cured by cooling over 2 hours at room temperature, and then gently removed by deforming the soft silicone mold.

6. 10:1 PDMS was gradually poured over the sugar mold, completely covering the surface, partially cured at ~50°C for 25-30 minutes. This was repeated five times. The PDMS was then fully cured for 12-18 hours at ~50°C in a dehydrating oven.

7. The cast PDMS with the sugar was positioned in the 3D printed crystal ribcage support and attached with dabs of glue.

8. The final assembly was left in lukewarm water for 2-3 hours to dissolve the cast sugar to obtain a clear PDMS crystal ribcage.

C. Rigid polystyrene ribcage fabrication

1. The 3D printed insert was positioned under the dental vacuum forming device.

2. 0.7mm clear polystyrene sheet (5” x 5”) was locked in the device jaw and heated till it first sags.

3. The heating element was turned off and the sheet was pulled over the 3D printed insert while applying a vacuum to remove all the trapped air between the mold and the polystyrene.

4. The formed polystyrene sheet with the 3D insert was removed from the vacuum former and extra polystyrene was trimmed around the edge.

5. The polystyrene was punctured at the neck of the 3D insert with the 1/8” circular file and press fit into a 3D printed support neck using the 5/32” file to complete the rigid crystal ribcage.

D. Crystal ribcage surface treatment
1. Clean the surface of the crystal ribcage with ethanol, rinse thoroughly with DI water, and dry the surface completely before proceeding with the steps below.

Hydrophilic engineering of PDMS crystal ribcage surface as previously described \(^{21}\)

2. 1% PDMS-PEG Block Copolymer v/v was combined with 10:1 PDMS and allowed to degas over 30 minutes in a 4°C fridge

3. The degassed PDMS-PEG solution was poured along the internal surface of the PDMS crystal ribcage and allowed to flow over the entire area.

4. The coated crystal ribcage was placed in the dehydrating oven at ~50°C for 8 hours to reach full cure

Hydrophilic engineering of polystyrene crystal ribcage surface

5. The polystyrene crystal ribcage was treated with oxygen plasma using a Harrick Plasma Cleaner.

6. The crystal ribcage was subjected to a vacuum of 800-900 mTorr and treated at medium power for 2 minutes in the plasma cleaner.

**Troubleshooting**

**Time Taken**

The time taken for each step varies along with its frequency in the fabrication process. This is described in detail in Table 1. The deformable PDMS crystal ribcage is significantly more time consuming taking up to almost 24 hours while the rigid polystyrene crystal ribcage can be fabricated more simply in under 30 minutes.

**Anticipated Results**

The final crystal ribcage fabricated should be a transparent, biocompatible platform to provide a physiological environment for a functioning lung *ex vivo* and allows multiscale optical imaging of the lung in health and disease. The internal surface of the crystal ribcage should be hydrophilic, so that the lung slides freely along it in its natural configuration during ventilation, to provide the same surface properties as the native parietal pleura in the mouse chest cavity. The fabricated crystal ribcage should be 150 μm thick (similar to a glass coverslip) for imaging with high-powered objectives (e.g., 60x), and the ribcage's curved geometry should not cause optical aberrations. The rigid crystal ribcage closely mimics the *in vivo* ribcage in quiet breathing as the majority of the lung ventilation is performed by diaphragm \(^{22}\). PDMS generates a soft crystal ribcage that is partially deformable to mimic the macroscale ribcage deformation that occurs *in vivo* during exercise, sighs, and coughs. Additionally, the PDMS ribcage is
pierceable and self-healing to enable intraparenchymal injections. Finally, the fabricated crystal ribcage does not affect the alveolar respiratory function compared to the intact mouse ribcage.

References


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**Figures**
<table>
<thead>
<tr>
<th>Procedure</th>
<th>Time (in hours)</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>A 1-2</td>
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<td>X</td>
</tr>
<tr>
<td>A 3</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>B 1-3</td>
<td>1.5</td>
<td>7</td>
</tr>
<tr>
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<td>2</td>
<td>24</td>
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<tr>
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<td>6</td>
</tr>
<tr>
<td>C 1-6</td>
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</tr>
<tr>
<td>D 5-6</td>
<td>&lt; 0.1</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Time taken with each step of fabrication of the either crystal ribcage

Figure 1

Time taken to complete different steps of the procedure