**Cubic clearing protocol for lung**

Dissection: Perfuse the animal with 1xPBS to clear blood from lungs. Gravity inflate lungs with 4% PFA and fix overnight rotating at 4 degrees.

Wash: Wash lungs at least 3x 10 minutes with 1xPBS rotating at RT. Separate lobes so you can image them individually after clearing.

Clearing: Rotate lungs at RT in Cubic R1 buffer for at least 1 week. I clear multiple lung lobes in a 15 mL tube filled with R1 buffer. May need to add fresh buffer to sample if the buffer starts turning yellow/green. Clearing can also be done at 4 degrees but it will take longer. Can move sample to 4 degree room after 1 week to continue clearing until you are ready to image or to store the sample in R1 buffer.

Prepare for lightsheet imaging:

-The day before imaging, embed cleared lung sample in 3% low melt agarose. Once the agarose block has hardened, trim the block so that it is as small as possible for imaging.

-Attach staples with superglue if needed for suspending in lightsheet chamber.

-Store the agarose block with staples attached in R2 buffer overnight so that the agarose meets the refractive index of the R2 buffer by the time of imaging.

Imaging (Zeiss Z1 Lightsheet)

-Fill lightsheet chamber with R2 buffer and suspend sample by attaching a magnet to the sample holder, and using the magnet to hold the staples and agarose block.

-To get the largest view of the sample, we use a 2.5x objective with a large imaging chamber manufactured by Translucence Biosystems (Mesoscale Imaging System).

-For long term storage, place samples back in R1 buffer (do not leave in R2 buffer for long periods of time).

**Susaki et al Nature Protocol on Cubic clearing method: https://www.nature.com/articles/nprot.2015.085**

**CUBIC Solutions from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6168389/**

**CUBIC R1 - 500g; ~420mL**

1. Mix 125g of Urea (Sigma-Aldrich, cat. no. U5378) and 175mL of H2O in a glass beaker.
2. Stir on a hot plate over low heat or place in a water bath, up to 56 degrees Celsius, until the urea dissolves.

*Allowing the mixture to reach a temperature of up to 56 degrees, will facilitate other components going into solution, but this step is not necessary.*

1. Add 123g (or 124mL) of Quadrol (*N*,*N*,*N*′,*N*′-Tetrakis(2-Hydroxypropyl)ethylenediamine, Sigma-Aldrich, cat. no. 122262).

*Quadrol is very viscous, therefore, it should be weighed directly into the urea solution. If the volume must be measured by volume, heat the Quadrol to 56 degrees in a water bath prior to pouring.*

1. Stir over low heat until the Quadrol dissolves.
2. Add 70mL of TritonX-100 (Fisher, cat. no. BP151-500).
3. Remove from heat and stir until dissolved.

*Store the solution sealed at room-temperature. The shelf-life is approximately 1 month. When the solution takes on a strong ammonia smell, it has expired. If the temperature is too high when making the solution, the ammonia smell will be immediately present, and the solution should be discarded.*

**CUBIC R2 - 500g; ~380mL**

1. Mix 125g of Urea (Sigma-Aldrich, cat. no. U5378) and 75mL of H2O in a glass beaker.
2. Stir on a hot plate over low heat or place in a water bath, up to 56 degrees Celsius, until the urea dissolves.

*Allowing the mixture to reach a temperature of up to 56 degrees, will facilitate other components going into solution, but this step is not necessary. The container should remain loosely capped to limit evaporation.*

1. Slowly add 250g of sucrose (Sigma-Aldrich, cat. no. S9378) with stirring over low heat.
2. Stir until dissolved using low heat. When dissolved, the solution will be extremely viscous.
3. Turn off heat and add 44.5mL of Triethanolamine (TEA) with stirring.
4. Add 380μL of TritonX-100 until well mixed.

*Store the solution sealed at room-temperature. The shelf-life is approximately 1 month. When the solution develops a strong ammonia smell, it has expired. If the temperature is too high when making the solution, the ammonia smell will be immediately present, and the solution should be discarded.*