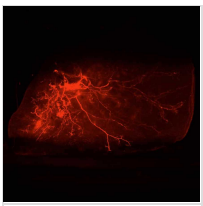

Dec 10, 2021

Protocol for CUBIC Clearing and Whole Mount Imaging of Mouse Lung Lobes

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This protocol is for Clear, Unobstructed Brain/Body Imaging Cocktails and Computational analysis (CUBIC) of mouse lung tissue for whole lobe imaging using Zeiss Lightsheet Imaging.

All experimental procedures were performed in the American Association for Accreditation of Laboratory Animal Care (AAALAC)-certified laboratory animal facility at the University of California San Diego, following protocols approved by the institutional animal care and use committee (IACUC). The procedures should incorporate all local requirements for standards of animal experimentation.

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Subjects:

A	B	C	D
Species	**Strain**	**RRID for strain**	**Supplier**
Mus musculus	Vglut2-ires-cre	IMSR_JAX:028863	The Jackson Laboratory
Mus musculus	Rosa-lxl-tdTomato	IMSR_JAX:007914	The Jackson Laboratory
Mus musculus	Nkx2-1 GFP	MMRRC_066764-JAX	Gift from Dr. D. Kotton and Dr. L. Ikonomou

The *Cre* lines used in the experiments were kept in B6 background. *Cre* males were mated to B6 wild-type females and both male and female mice were used in the experiment.

CUBIC R1 buffer:

- Urea - Sigma-Aldrich, Catalog #U5378
- Quadrol (N,N,N',N'-Tetrakis(2-Hydroxypropyl)ethylenediamine) - Sigma-Aldrich, Catalog #122262
- TritonX-100 - Fisher, Catalog #BP151-500

CUBIC R2 buffer:

- Urea - Sigma-Aldrich, Catalog #U5378
- Sucrose - Sigma-Aldrich, Catalog #S9378
- TritonX-100 - Fisher, Catalog #BP151-500

Light Sheet Microscopy:

- Light sheet fluorescence microscope -Zeiss Z.1, Zeiss
- Translucence Specimen Holder - Translucence Biosystems, Mesoscale Imaging System

Clearing preparations

1 Euthanize mice by CO_2 inhalation.

1.1 To clear blood from the lungs perfuse transcardially the animals with 1xPBS. Gravity inflate lungs with 4% paraformaldehyde (PFA) and fix overnight at **4 °C** .

1.2 Wash the lungs at least 3x for 10 minutes with 1xPBS rotating at **Room temperature** .

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1.3 Separate lobes to image them individually after cleaning.

Clearing procedures

2 Rotate the lungs at **Room temperature** in CUBIC R1 buffer for at least 1 week.

- Multiple lung lobes can be cleared in a **15 mL** tube filled with R1 buffer.
- If the buffer starts turning yellow or green add a fresh buffer to the sample.
- Clearing can be done at **4 °C** but will take longer.

Clearing procedures were done according to Susaki et al., (2015)

2.1 After 1 week move samples to **4 °C** room to continue clearing until you are ready for imaging or to store the samples in CUBIC R1 buffer.

Clearing Solutions

3 **CUBIC buffers** *were prepared according to Muntifering et al., (2018)*

4 **CUBIC R1** - **500 g** / ~ **420 mL**

4.1 Mix **125 g** of Urea (Sigma-Aldrich) and **175 mL** H_2O in a glass beaker.

4.2 Stir on a hot plate over low heat or place in a water bath, up to **56 °C** , 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*.

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4.3 Add **123 g** (or **124 mL**) of Quadrol (Sigma-Aldrich).

> 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 °C** in a water bath prior to pouring.

4.4 Stir over low heat until the Quadrol dissolves.

4.5 Add **70 mL** of TritonX-100 (Fisher, cat. no. BP151-500).

4.6 Remove from heat and stir until dissolved.

4.7
- *Store the solution sealed at* **Room temperature** *for 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.*

5 CUBIC R2 - 500 g / ~ 380 mL

5.1 Mix **125 g** of Urea (Sigma-Aldrich) and **75 mL** H_2O in a glass beaker.

5.2 Stir on a hot plate over low heat or place in a water bath, up to **56 °C** , until

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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.

5.3 Slowly add **250 g** of sucrose (Sigma-Aldrich) with stirring over low heat.

5.4 Stir until dissolved using low heat. When dissolved, the solution will be extremely viscous.

5.5 Turn off heat and add **44.5 mL** of Triethanolamine (TEA) with stirring.

5.6 Add **380 µL** of TritonX-100 until well mixed.

5.7
- *Store the solution sealed at* **Room temperature** *for 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.*

Light sheet imaging preparation

6 One day before imaging:

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6.1 Embed cleared lung sample in 3% low melt agarose.

6.2 Once the agarose block has hardened, trim the block as small as possible for imaging.

6.3 If needed, attach staples with superglue for suspending in light sheet chamber.

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

Imagining

7 Imaging was done with Zeiss Z.1 Lightsheet

7.1 Fill light sheet chamber with CUBIC R2 buffer.

7.2 Suspend the sample by attaching a magnet to the sample holder and using the magnet to hold the staples and agarose block.

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

7.4 For long term storage place the sample back in the CUBIC R1 buffer. Do not leave the sample in the CUBIC R2 buffer for a long time.

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