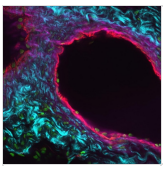

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622.2 URMC HTC Rapid Clearing of Thick Human Lung Tissue Sections

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1 Works for me dx.doi.org/10.17504/protocols.io.7d7hi9n

Human Cell Atlas Method Development Community LungMap2 Consortium 1 more workspace

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ABSTRACT

Purpose and Scope of the Procedure:
Provide a relatively rapid clearing of lung tissue sections up to 1 millimeter thick for immunofluorescence staining and imaging with confocal or multiphoton microscopy.
Current protocol is limited to tissue sections that are free from severe hemorrhage/thrombosis, containing mostly parenchyma, vessels, and smaller bronchioles. Times and solution volumes may need to be altered slightly if one wishes to clear sections containing a lot of cartilage and/or blood.

Scientific Principles: One of the major challenges in conventional brightfield, epifluorescence, and even confocal microscopy is the limitation in imaging depth. This is primarily due to the scattering and absorption of light within the sample of both the excitation light and the emission light leading to a drastic reduction in the image brightness and contrast when deeper than a 100µm. Clearing offers a way around this by both reducing the components that negatively impact the light and by matching the refractive index of the mounting medium to that of the components which make up the tissue, leading to an almost transparent appearance.

This protocol is a slight modification of a Clear Unobstructed Brain/Body Imaging Cocktail (CUBIC) protocol described by Kubota et al. developed originally to allow the analysis of cancer metastasis within the bodies of entire mice. By taking advantage of the fact lung tissue has plenty of open spaces and a very large surface area we can reduce the time in each of the solutions to allow complete clearing of the tissue sections to within just a few days.

References:
·Light Microscopy Methods and Protocols (2017). p. 74
·Kubota et al., (2017). Whole-Body Profiling of Cancer Metastasis with Single-Cell Resolution.Cell Reports 20, 236–250

DOI

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KEYWORDS

CUBIC

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MATERIALS

NAME	CATALOG #	VENDOR
N-butyldiethanolamine	B0725	TCI Chemicals
N-methylnicotinamide	M0374	TCI Chemicals
Antipyrine	104971000	Acros Organics
Triton X-100	X100-100ML	Sigma Aldrich
10X Phosphate Buffered Saline	46013CM	Corning

MATERIALS TEXT

· One (1) 50 milliliter conical tube per sample, alternatively one (1) 35 millimeter plate per sample
· Four (4) 50 milliliter conical tubes to prepare and store the CUBIC solutions.
· Two (2) 50 milliliter conical tubes to store the used CUBIC-R solutions.
· A test tube rack capable of holding 50 milliliter conical tubes or a small plastic tray to place 35 millimeter plates on (if applicable)
· Incubator capable of maintaining 37°C overnight
· Plastic transfer pipettes (fine tipped)
· A balance capable of measuring to at least the nearest 0.01 grams.
· One (1) 2 milliliter glass pipette
· Millipore ultrapure water system
· Parafilm M
· Aluminum Foil
· Rotator capable of running continuously
· Silicone vacuum grease
· 3mL syringe with luer lock
· Small (18G-25G) blunt tipped cannula with luer lock
· 100mm plate (or disposable glass slide if this is preferable)
· Glass Coverslips #1
· 2 fine tipped curved forceps

SAFETY WARNINGS

All work should be performed with proper PPE including gloves, lab coat, and close-toed shoes. In addition, a fume hood should be utilized when preparing solutions. It is also very important that the cannula used when preparing the wells during the final steps is a blunt tipped cannula to avoid the possibility of injury.

BEFORE STARTING

Sections of lung between **500 µm** and **1 mm** should be cut and immediately processed or kept frozen (or cold, if cut with a vibratome) until processing begins. If not previously fixed with either formalin or paraformaldehyde then these tissue sections should be placed into fixative overnight before any mounting solution is washed away (if applicable).

Preparing Reagents

1 **1X Phosphate Buffered Saline**

Add **50 mL** of 10X Phosphate Buffered Saline to **450 mL** ultrapure water and mix the solution well. Store at room temperature.

100% CUBIC-L

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Add 16g of ultrapure water to a 50mL conical followed by **2 g** of Triton X-100. Swirl the tube until the Triton X-100 completely dissolves into solution. Next, in a fume hood, add 2mL N-butyldiethanolamine to the solution and swirl until the solution is homogeneous. Place Parafilm around the cap to avoid any evaporation and store at room temperature.

50% CUBIC-L

In a separate 50mL conical tube, add **5 mL** of ultrapure water and **5 mL** of 100% CUBIC-L solution. Swirl until the solution is properly mixed. Place Parafilm around the cap to avoid any evaporation and store at room temperature.

100%CUBIC-R

In a 50mL conical tube add **5.0 g** of ultrapure water followed by **6.0 g** of N-methylnicotinamide. Close and shake the tube until all the N-methylnicotinamide is dissolved. Finally, add **9.0 g** of Antipyrine to the tube and shake until completely dissolved. The solution should have a clear or slight yellow appearance to it. This solution is very susceptible to precipitation so Parafilm must be placed around the cap immediately after each use to avoid any evaporation. Store at room temperature.

50% CUBIC-R

In a separate 50mL conical tube, add **5 mL** of ultrapure water and **5 mL** of 100% CUBIC-R solution. Swirl until the solution is properly mixed. Place Parafilm around the cap to avoid any evaporation and store at room temperature.

Clearing the Tissue

2 Label the 50mL conical tubes to be used with the appropriate section ID and fill each tube with **40 mL** 1X Phosphate Buffered Saline (PBS). If the PBS is already a 1X solution you may ignore this step.

3 Add sections in their respective 50mL conical tube, placing the tube into a rack and onto the rotator at a low speed for 15 minutes. Repeat this step 4 more times for each tube bringing the total number of PBS washes to 5. Use a transfer pipette to remove and add fresh PBS making sure to not damage the sections.

4 After the 5th wash, add another **40 mL** PBS and leave on the rotator at a low speed while preparing for the next step.

5 Remove the PBS from the sample using a transfer pipette making sure to not damage the section in the process. Immediately add **5 mL** of 50% CUBIC-L and gently swirl to verify the section is completely free floating. Place the 50mL conical tube into a rack. Repeat this step for all samples.

6 When the previous step is completed, place the rack into a **37 °C** incubator for a minimum of 3 hours (maximum 6 hours). Ideally the sections would be on a rotator at a low speed, but if this is unavailable then gently swirl the sections once or twice throughout the incubation if possible.

7 Remove the sections from the incubator and remove the 50% CUBIC-L solution with a transfer pipette, ensuring there is no damage to any of the sections.

7.1 Dispose of the used 50% CUBIC-L solution into a properly labeled waste container.

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7.2 Immediately after removing the 50% CUBIC-L solution from a section add 5mL of 100% CUBIC-L solution and giving the tube a gentle swirl and verifying the section is free floating.

7.3 Once each day, replace the used 100% CUBIC-L solution with fresh solution and place the samples back into the incubator. Add the removed 100% CUBIC-L to the waste container.

7.4 Place the samples into a 37°C incubator.
The samples will be in CUBIC-L solution for a minimum of 2 days. This time may be longer depending on how much blood the tissue contains.

8 At the end of 2 days (or when clearing is determined to be sufficient, see analysis of results) remove the samples from the incubator and gently remove the 100% CUBIC-L solution.

8.1 Wash the samples with 40mL PBS on the rotator, 5 times for 15 minutes each.

8.2 After the 5th wash replace with a fresh 40mL PBS. At this stage you may temporarily store the sections overnight in a cold room.

9 The samples are ready to be immunofluorescently stained.
No more Triton X-100 should be used from this point on since it can have a negative impact on staining.

Refractive Index Matching

10

The following assumes that you have completed any fluorescent staining and have completed the final wash.

After immunofluorescent staining is complete, from each section remove the last wash buffer with a transfer pipette and replace with **2 mL** of 50% CUBIC-R solution per section. The sections should be placed on a rotator at a low speed for at least 2 hours at room temperature and in the dark.

11 Remove the 50% CUBIC-R and immediately replace with **2 mL** of 100% CUBIC-R. Place these on the rotator at a low speed overnight (or a minimum of 4 hours).

11.1 Place the recovered 50% CUBIC-R into a 50mL conical tube labeled for **used** 50% CUBIC-R solution to catch any sections that may be accidentally suctioned up with the solution. Once it is determined that all the sections are present in their respective wells this used 50% CUBIC-R solution can be disposed of in a properly labeled waste container.

12 Add **1 mL** vacuum grease to a 3mL syringe and attach the small cannula. In a 100mm plate (or disposable glass slide if this is preferable), use this cannula to add a small ring of silicone grease a little larger than the tissue section to be mounted. Using a marker, make a label on the plate just next to the well that was previously created.

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13 Using 2 fine tipped forceps, gently grab a cleared tissue section by the 2 corners along the same edge to avoid folding of the section and gently place it in the previously made well. Smooth out the section as much as possible gently with the forceps after placing in the created well within the 100mm plate.

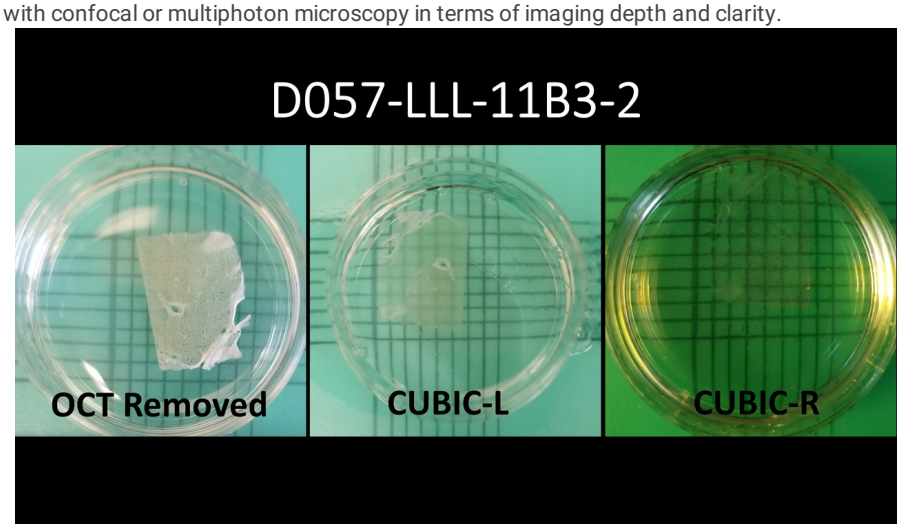
14 On top of this section add 1-2 drops of fresh 100% CUBIC-R solution. Gently work out any air bubbles formed during this process use the forceps.

15 Gently place the glass coverslip over the section starting at an angle to avoid the formation of any air bubbles. Gently press the glass coverslip to ensure the glass and vacuum grease are sealed and no mounting medium is leaking. The section is now ready for imaging.

16 Use a transfer pipet to move the used 100% CUBIC-R solution from the 6 well plate into a properly labeled waste container.

Analysis of Results

17 A tissue section that has been perfectly cleared with this method should be almost completely transparent and around the same size as it was at the beginning of the clearing process. If the tissue section is in a clear bottom plate, position the plate over a sheet of paper with some text. ideally, the text will be perfectly readable through the tissue with little to no distortion. This serves as a good quick determination of the clearing quality. The true test is the quality of imaging with confocal or multiphoton microscopy in terms of imaging depth and clarity.



Left: Section of lung immediatly after removal of OCT via washes with PBS. Middle: At the end of CUBIC-L treatment, the sections should be completely, or near completely, colorless. The section is still visible, but noticably more transparent. Right: (After steps 10-12) When adding the 100% CUBIC-R solution, the section will become transparent. Notice in the image that the section is still barely visible. After being allowed to incubate overnight the section will become even more transparent and care must be taken when handeling since it will be extremely difficult to see.

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