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Tissue clearing of mouse organs and zebrafish using CUBIC L / CUBIC HL and CUBIC RA reagents V.1

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We use this protocol and it's working

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Disclaimer

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Abstract

The present protocol describes optical tissue clearing of murine and zebrafish tissue with CUBIC L or CUBIC HL delipidation reagents and CUBIC RA refractive index matching reagent (developed by [Tainaka et al., 2018](#)), with adaptations and modifications. The present protocol does not describe tissue clearing with ScaleCUBIC-1 and ScaleCUBIC-2 reagents of the 1st version of the CUBIC method ([Susaki, Etsuo A. et al., 2014](#)).

The present protocol covers the steps of solution preparation, tissue fixation, delipidation, refractive index matching, optional nuclear staining and embedding the samples in agarose for image acquisition on light sheet fluorescence microscope. Handling and manipulations with live animals as well as light sheet image acquisition and analysis are beyond the scope of the present protocol. The present protocol permits tissue clearing, decolorization and fluorescent taining of the nuclei of various murine tissues (brain, liver, kidney, intestine, thymus, lymph nodes and others) and whole body adult *Danio rerio*, thus making these samples suitable for 3D LSFM imaging, using fluorescent signals of fluorescent proteins (except samples treated with CUBIC HL reagent) and/or those of the nuclei stains (propidium iodide or SYBR Gold). Immunofluorescent staining, which can be combined with CUBIC tissue clearing ([Tainaka et al., 2018](#)), is not described in the present protocol.

Materials

Reagents

1. Paraformaldehyde (PFA) (for example, AlfaAesar)
2. N-butyldiethanolamine (Sigma-Aldrich)
3. Triton X-100 (Nacalai Tesque)
4. Antipyrine (Tokyo Chemical Industry)
5. N-methylnicotinamide (Tokyo Chemical Industry)
6. phosphate buffered saline (PBS), pH 7.4 (for example, GIBCO)
7. 1,3-bis(aminomethyl)cyclohexane (Tokyo Chemical Industry)
8. sodium dodecylbenzenesulfonate (Tokyo Chemical Industry)
9. *p*-toluenesulfonic acid (Sigma-Aldrich)
10. Propidium iodide (Thermo Fischer Scientific)
11. SYBR Gold (Thermo Fisher Scientific)

Equipment:

1. Rocking platform
2. pH-meter
3. 5 ml polypropylene Eppendorf tubes
4. A temperature-controlled room or cabinet which permits to thermostate the rocking platform at 37 degrees throughout the incubations which may last multiple days
5. Non-sharp forceps or spatula
6. Standard polystyrene 12-well or 6-well plates
7. 1-ml polypropylene "tuberculin" syringe without needle (for example, Becton Dickinson)

Optional equipment:

1. Scalpels
2. Steel or plastic dissection board
3. Parafilm
4. Refractometer
5. Vacuum pump
6. Digital photcamera which can focus at the samples (to document progress of clearing)
7. Millimetre paper



Safety warnings

- ! Handle all the chemicals with care and use appropriate personal protective equipment (PPE), according to manufacturer's instructions and your local regulations.
Follow institutional guidelines for the disposal of hazardous materials.

Ethics statement

Experiments involving animals must be conducted according to internationally-accepted standards and should always have prior approval from ethics committee(s) according to legislation of your country. Please indicate that prior ethics approval should be obtained before performing these experiments.

Before start

Handle all the chemicals with care and use appropriate personal protective equipment (PPE), according to manufacturer's instructions and your local regulations
Follow institutional guidelines for the disposal of hazardous materials.



Preparation of the working solutions:

1 Fixative solution

- 1.1 Dilute commercial stock solution of paraformaldehyde in PBS to final concentration of 4% (w/w) and store the aliquotes at -20°C.

2 CUBIC L (delipidation solution 1)

- 2.1 Place N-butyldiethanolamine (10% of the target weight of the final solution), Triton X-100 (10% of the target weight of the final solution) and water (80% of the target weight of the final solution) in a suitable container
- 2.2 Tightly close the container and mix on a rocking platform at a slow rocking rate (below 10 rocking motions per min.) until the solution becomes transparent.

Note

Do not vortex the mixture. Do not sonicate the mixture.

3 CUBIC HL (delipidation solution 2)

- 3.1 Place 1,3-bis(aminomethyl)cyclohexane (10% of the target weight of the final solution), sodium dodecylbenzenesulfonate (10% of the target weight of the final solution) and water (<80% of the target weight of the final solution) in a suitable container
- 3.2 Tightly close the container and mix on a rocking platform at a slow rocking rate (below 10 rocking motions per min).

Note

Do not vortex the mixture. Do not sonicate the mixture.

- 3.3 Adjust pH of the mixture to 12.0 with 10 M solution of *p*-toluenesulfonic acid



- 3.4 Add the amount of water required to reach the target weight of the final solution
- 4 CUBIC RA (The reagent for refractive index (RI) matching and light sheet image acquisition)
 - 4.1 Place antipyrine (45% of the target weight of the final solution), N-methylnicotinamide (30% of the target weight of the final solution)) and water (< 25% of the target weight of the final solution) in a suitable container
 - 4.2 Tightly close the container and mix on a rocking platform at a slow rocking rate (below 10 rocking motions per min) until the solution becomes transparent.
 - 4.3 If nuclear staining with propidium iodide or SYBR Gold is planned, then add propidium iodide to CUBIC RA solution to final concentration of 10 mkg/ml, or dilute the commercial SYBR Gold stock solution 1:1000 in CUBIC RA and mix on a rocking platform

Note

Light sheet image acquisition should be performed in a dye-free refractive index matching solution, so nuclei stains should not be added to the aliquot of CUBIC RA solution which will be used to fill the light sheet microscope sample chamber.

Fixation

- 5 Place the dissected murine organ or fragment of tissue in a well of 12-well or 6-well plate on ice
- 6 Rinse the sample with cold PBS on ice to remove residual blood. Repeat rinsing with PBS until it is not getting colored with blood anymore.
- 7 Replace PBS with the fixative solution (4% PFA) and incubate overnight at +4°C in darkness
- 8 Move samples from fixative solution to cold PBS and rinse with cold PBS several times
- 9 Cut the organ or tissue into the pieces using a scalpel on a dissection board.





Note

Cutting is only recommended if it does not compromise the morphology of the structure of interest. The thickness of the sample strongly influences the time required for delipidation, refractive index matching and staining steps. Moreover, the larger the sample, the more difficult mounting in LSMF sample chamber and acquisition of the images is. Therefore, although the present protocol is suitable for clearing entire adult mouse organs, such as brain or kidney, we recommend cutting organs into pieces if this would not compromise the morphological features of interest.

Delipidation

- 10 Put the sample in the well of 6-well or 12-well plate, add PBS, place millimeter paper under the plate, take a photograph

Note

This is an optional step, recommended for documentation purposes

- 11 Add 3 ml of pre-warmed CUBIC L solution or CUBIC HL solution into a 5 ml Eppendorf test tube

Note

CUBIC L and CUBIC HL solutions are the two options which we used for delipidation. In the original publication (Susaki et al 2018), CUBIC HL was used to clear large fragments of human tissue. We observed that murine tissues which are not optimally cleared in CUBIC L can be cleared more efficiently with CUBIC HL. Thus we recommend to use CUBIC HL in such cases

Note

CUBIC HL bleaches fluorescent proteins, so this reagent should not be used if emission of a fluorescent protein will be collected

- 12 Put each sample into individual 5 ml Eppendorf tube containing 3 ml of CUBIC L or CUBIC HL solution.



Figure 1. A fragment of murine liver prepared for delipidation step

- 13 Fix the 5-ml Eppendorf tubes on a rocking platform (kept at $+37^{\circ}\text{C}$) in a tilted or horizontal position.
- 14 Switch on slow rocking (below 60 rocking motions per min), keep the rocking platform at $+37^{\circ}\text{C}$; protect the samples from light
- 15 Every day, visually inspect each tube with the sample. If CUBIC L or CUBIC HL solution in the tube becomes colored or turbid, then replace it with fresh pre-warmed aliquot of CUBIC L or CUBIC HL respectively. Optionally take a photograph as described in the step 10. When the sample gets partially transparent, then proceed to the next step.

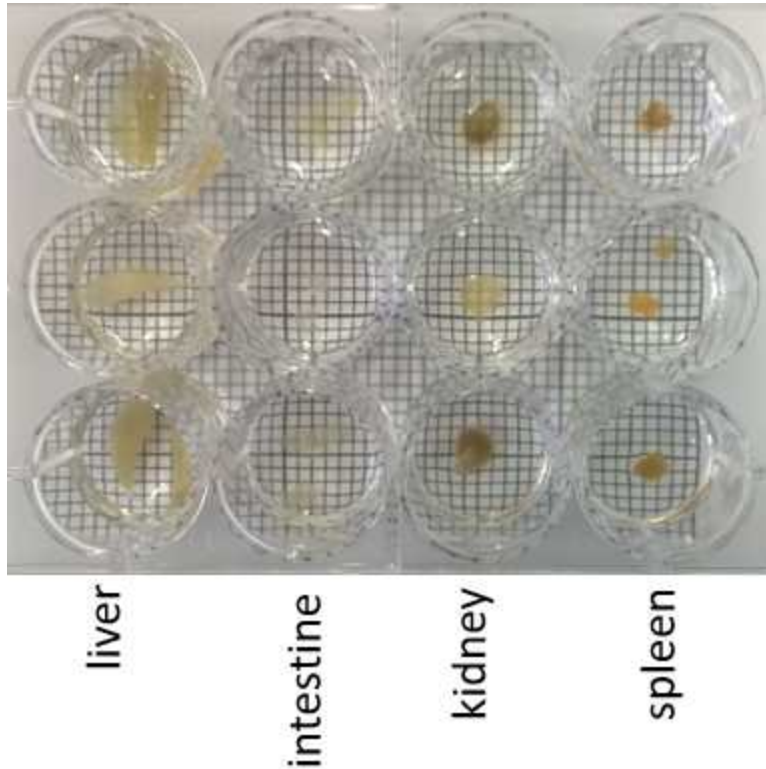


Figure 2. Expected result: fragments of murine organs after 9 days of delipidation.

Refractive index matching (optionally combined with nuclei staining)

- 16 Transfer the sample to PBS (pH 9.15)
- 17 Place sample in a well of 12-well plate, put the plate on millimeter paper, take a photograph
- 18 Rinse the sample in PBS (pH 9.15) 3 times, 15 min each time, on a rocking platform
- 19 Put the sample in a 5-ml Eppendorf tube containing 3 ml of pre-warmed CUBIC RA solution which may optionally contain propidium iodide or SYBR Gold, if staining of the nuclei is needed
- 20 Fix the Eppendorf tubes on a rocking platform in a tilted or horizontal position



- 21 Switch on slow rocking (below 60 rocking motions per min), keep the rocking platform at +37°C; protect the samples from light
- 22 Visually inspect the tubes with samples every day. If CUBIC RA solution became colored or opaque, then discard it, add fresh warm CUBIC RA aliquot and continue incubation as described in the step 21. Optionally take a photograph as described in the step 10. If the sample appears transparent, then proceed to the next section. If the sample does not appear transparent, then continue incubation as described in the step 21.

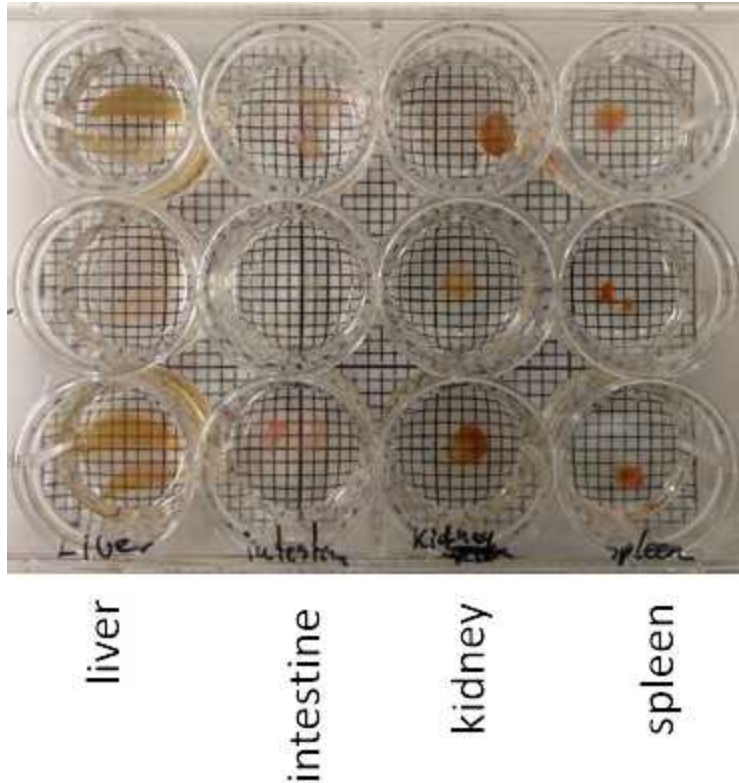


Figure 3. Expected result. Fragments of murine organs (the same samples as on the Figure 2) after delipidation and refractive index matching combined with propidium iodide staining.

- 23 If a nuclear stain (propidium iodide or SYBR Gold) was present in CUBIC RA solution at the steps 19-22, then transfer the sample to dye-free CUBIC RA solution and incubate overnight at room temperature

Embedding samples in agarose for image acquisition on Zeiss Lightsheet.Z1 microscope

- 24 Cut the needle adapter off the barrel of 1 ml tuberculin syringe, such that the remaining part of the barrel has uniform diameter (as shown on the picture)

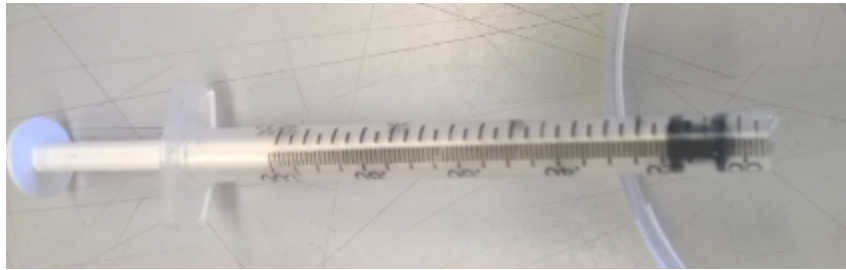


Figure 4. 1 ml syringe with needle adapter cut off, which can be used for embedding the samples in agarose

- 25 Dissolve agarose in PBS (pH 7.4), to obtain 2% solution (w/w).

Note

Do not dissolve agarose in CUBIC RA solution

- 26 Put approximately 5 ml of the 2% agarose solution into the 15-ml falcon tube
- 27 Put the sample into the tube with the mentioned solution while it is still liquid
- 28 Aspirate the sample together with agarose solution into the 1 ml syringe pre-cut as shown on Figure 4
- 29 Leave the syringe at +4°C in darkness until agarose solidifies (The syringe serves as a mold in which cylinder of agarose will be formed)
- 30 Prepare a test tube whose inner diameter is several mm larger than the outer diameter of the 1 ml syringe (for example, 15 ml Falcon tube) half-filled with CUBIC RA solution
- 31 Immerse the syringe with agarose-embedded sample into the tube containing CUBIC RA solution and fix the syringe such that its tip is at least 1 cm above the bottom of the tube, as shown on Figure 5



- 32 Push the plunger such that the cylinder of solidified agarose with the embedded sample partially exits the syringe and becomes immersed in CUBIC RA solution

Note

Do not push the agarose cylinder completely out of the syringe. The cylinder should "hang" below the syringe

- 33 Seal the tube with syringe using Parafilm to minimize evaporation



- 34 Leave the syringe with the sample in CUBIC RA at room temperature until the agarose cylinder becomes nearly invisible (overnight or longer)



Figure 5. Syringe with a cleared sample embedded in agarose, immersed in CUBIC RA solution.

- 35 When the agarose cylinder becomes transparent, pull it inside the syringe and proceed to installation of the syringe in the light sheet microscope sample holder for image acquisition

Note

When the sample is mounted as described above, it can be stored (before or after image acquisition) for at least several days, at +4°C, protected from light

Protocol references

1. Chemical Landscape for Tissue Clearing Based on Hydrophilic Reagents. Tainaka, Kazuki et al. Cell Reports, Volume 24, Issue 8, 2196 - 2210.e9. DOI: [10.1016/j.celrep.2018.07.056](https://doi.org/10.1016/j.celrep.2018.07.056)
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