Protocol Booklet

Product Code(s)	HB8771
Product Name	Tissue Clearing Kit
Purpose	Clearing of tissue and/or organoids for volumetric imaging

Please note: This product is for RESEARCH USE ONLY and is not intended for therapeutic or diagnostic use. Not for human or veterinary use

Contents

Product Overview

This step by step protocol provides everything you need to successfully clear tissue and organoids using the Hello Bio Tissue Clearing Kit (HB8771). Written by our PhD qualified expert team, this protocol is suitable for a range of different tissue and organoid types of differing sizes and includes comprehensive troubleshooting information to head off any potential problems.

Sample Type	Fixation time	Total Clearing Time
1 mm tissue	≈12 hours	≈1 day
3 mm tissue	≈12 hours	≈5 days
3 - 7 mm tissue	≈24 hours	≈5 days
≥7mm tissue	≈24 hours	≈8 days
Organoid	≈30 minutes	≈1 day

Table 1. Representative clearing times (no immunostaining) for different types of sample

We have some general advice to maximise the chance of success when conducting tissue clearing:

- If possible, the smaller the amount of tissue to be cleared the better. Where possible dissect out regions of interest.
- Don't worry if the sample clears during the process only to turn opaque again at a later step. After incubation in the mounting and storage solution this will reverse and the sample will become transparent again.
- For immunostaining the diffusion time of the antibodies is directly proportional to their size. If possible use antibody formats such as Sv-Fvs or nanobodies to increase the diffusion into a tissue sample.
- Try and ensure that there is minimal air in the tubes that the samples are incubated in to reduce sample oxidation.

Components & Storage

This kit contains:

- Tissue clearing solution A (30 ml)
- Tissue clearing solution B (60 ml)
- Antibody penetration buffer (100 ml)
- Mounting and Storage solution (100 ml)
- DAPI staining solution (30 ml)

Note: Store kit at -20°C until use.

This kit additionally requires:

- 4% paraformaldehyde
- Phosphate buffered saline (PBS)
- 30% sucrose in PBS
- Antibody penetration and blocking solution
    - Add serum to the provided antibody penetration buffer to a final concentration of 5%
- Antibody dilution buffer
    - Conduct a 1:1 dilution of antibody penetration buffer with PBS then add serum to a final concentration of 5%
- Heated shaking incubator
- Light sheet or confocal laser scanning microscope.

Clearing Protocol

Optimal clearing is achieved by varying the protocol to different sized sample types. It is therefore important to follow the protocol most closely describing your sample type.

Tissue: 1mm thickness

1. If possible trans-cardiac perfuse the animal with PBS then 4% PFA before then postfixing for 12-24 hours in 4% PFA at 4°C and then slicing into 1mm sections. See our IHC(IF) protocol for more information on perfusion fixation. Otherwise slice fresh tissue into 1mm sections then fix in 4% PFA for 12 hours at 4°C.
2. Wash the samples with PBS (3 x 20 minute washes at room temperature).
3. Incubate the samples with 6ml of 30% sucrose in PBS at 4°C until they sink (around 2 - 12 hours). For buoyant samples such as lung proceed to the next step after 12 hours.
4. Incubate the samples with 3ml of tissue clearing solution A at 37°C in a shaking incubator (50rpm) for 4-6 hours.
    a. Ensure that tissue clearing solution A has been incubated at 37°C for 1-2 hours before use and that any precipitates have redissolved.
5. Wash the samples with dH_2O at 37°C (50rpm, 4 x 30 minute washes)
6. Incubate the samples with 3ml of tissue clearing solution B at 37°C in a shaking incubator (50rpm) for 12 hours.
    a. Note: This step is often unnecessary and can be skipped if the tissue clearing is sufficient following incubation in tissue clearing solution A.
    b. Ensure that tissue clearing solution B has been incubated at 37°C for 1-2 hours before use and that any precipitates have redissolved.
7. Wash the samples with dH_2O at 37°C (50rpm, 4 x 30 minute washes)
8. Incubate the samples with 3ml of the DAPI staining solution and incubate at 37°C in a shaking incubator (50rpm) for 1 hour.
9. Wash the samples with PBS (6 x 20 minute washes, 37°C, 50rpm)
10. Incubate the samples with 5ml of mounting solution and incubate at 37°C in a shaking incubator (50rpm) for at least 12 hours.
11. The samples are now ready for imaging.

Tissue: 3-7mm thickness

1. If possible trans-cardiac perfuse the animal with PBS then 4% PFA before then postfixing for 12 hours in 4% PFA at 4°C and dissecting out the required tissue. See our IHC(IF) protocol for more information on perfusion fixation. Otherwise dissect fresh tissue then fix in 4% PFA for 12 hours at 4°C with agitation.
2. Wash the samples with PBS (3 x 20 minute washes at room temperature).
3. Incubate the samples with 15ml of 30% sucrose in PBS at 4°C until they sink (around a day). For buoyant samples such as lung proceed to the next step after 2 days.
4. Incubate the samples with 3-5ml of tissue clearing solution A at 37°C in a shaking incubator (50rpm) for 1 - 2 days (1 day for 3mm sections, 2 days for 7mm sections).
    a. Ensure that tissue clearing solution A has been incubated at 37°C for 1-2 hours before use and that any precipitates have redissolved.
5. Wash the samples with dH_2O at 37°C (50rpm, 4 x 30 minute washes)
6. Incubate the samples with 3-5ml of tissue clearing solution B at 37°C in a shaking incubator (50rpm) for 1-2 days.
    a. Ensure that tissue clearing solution B has been incubated at 37°C for 1-2 hours before use and that any precipitates have redissolved.
7. Wash the samples with dH_2O at 37°C (50rpm, 4 x 30 minute washes)
8. Incubate the samples with 5ml of the DAPI staining solution and incubate at 37°C in a shaking incubator (50rpm) for 6-12 hours (6 hours for 3mm sections, 12 hours for 7mm sections).
9. Wash the samples with PBS (6 x 20 minute washes, 37°C, 50rpm)
10. Incubate the samples with 10ml of mounting solution and incubate at 37°C in a shaking incubator (50rpm) for at least a day.
11. The samples are now ready for imaging.

Tissue ≥7mm thickness

1. If possible trans-cardiac perfuse the animal with PBS then 4% PFA before then postfixing for 24 hours in 4% PFA at 4°C. See our IHC(IF) protocol for more information on perfusion fixation. Otherwise dissect fresh tissue then fix in 4% PFA for 24 hours at 4°C with agitation.
2. Wash the samples with PBS (3 x 20 minute washes at room temperature).
3. Incubate the samples with 30ml of 30% sucrose in PBS at 4°C until they sink (around 2-4 days). For buoyant samples such as lung proceed to the next step after 4 days.
4. Incubate the samples with 5-10ml of tissue clearing solution A (10ml for a rat brain hemisphere, 5ml for a hippocampus) at 37°C in a shaking incubator (50rpm) for 2-3 days.
    a. Ensure that tissue clearing solution A has been incubated at 37°C for 1-2 hours before use and that any precipitates have redissolved.
5. Wash the samples with dH_2O at 37°C (50rpm, 4 x 30 minute washes)
6. Incubate the samples with 5-10ml of tissue clearing solution B at 37°C in a shaking incubator (50rpm) for 2-3 days.
    a. Ensure that tissue clearing solution B has been incubated at 37°C for 1-2 hours before use and that any precipitates have redissolved.
7. Wash the samples with dH_2O at 37°C (50rpm, 4 x 30 minute washes)
8. Incubate the samples with 5-10ml of the DAPI staining solution and incubate at 37°C in a shaking incubator (50rpm) for 24 hours.
9. Wash the samples with PBS (6 x 20 minute washes, 37°C, 50rpm)
10. Incubate the samples with 15ml of mounting solution and incubate at 37°C in a shaking incubator (50rpm) for at least 3 days.
11. The samples are now ready for imaging.

Organoids

1. Fix the organoids in 4% PFA for 1 hour.
2. Wash the samples with PBS (3 x 20 minute washes at room temperature).
3. Incubate the samples with 6ml of 30% sucrose in PBS at 4°C for 12 hours.
4. Incubate the samples with 3ml of tissue clearing solution B at 37°C in a shaking incubator (50rpm) for 4-24 hours (keep monitoring until the organoids are cleared).
    a. Ensure that tissue clearing solution B has been incubated at 37°C for 1-2 hours before use and that any precipitates have redissolved.
5. Wash the samples with dH_2O at 37°C (50rpm, 4 x 10 minute washes)
6. Incubate the samples with 3ml of the DAPI staining solution and incubate at 37°C in a shaking incubator (50rpm) for 1 hour.
7. Wash the samples with PBS (6 x 20 minute washes, 37°C, 50rpm)
8. Incubate the samples with 5ml of mounting solution and incubate at 37°C in a shaking incubator (50rpm) for at least 6 hours.
9. The samples are now ready for imaging.

Immunostaining Protocol

It is possible to stain cleared tissue sections and organoids using modified immunohistochemical approaches. Due to most commercially available antibodies not being validated in this application it is often necessary to characterise and optimise the process before further use. Additionally the thicker a tissue section then the slower and more difficult it is going to be for antibodies to diffuse into the tissue leading to elevated incubation times. This protocol is designed for 1mm thick tissue sections therefore all incubations will need extending if attempting to stain larger samples:

1. Incubate the samples with the penetration and blocking solution for 2-3 days in a shaking incubator (37°C, 50rpm).
2. Incubate the samples with the primary antibody diluted in antibody dilution buffer for 3-6 days in a shaking incubator (37°C, 50rpm).
    a. Generally higher antibody concentrations are required to stain cleared tissue. A good starting point for many antibodies is in the range 1:50 - 1:200 but it is recommended to optimize this.
    b. Antibody fragments or nanobodies will work better and reduce the incubation time required.
3. Wash the samples with PBS (6 x 20 minute washes, 37°C, 50rpm)
4. Incubate the samples with the secondary antibody diluted in antibody dilution for 3-6 days in a shaking incubator (37°C, 50rpm).
    a. Generally higher antibody concentrations are required to stain cleared tissue. A good starting point for many antibodies is in the range 1:100 - 1:500 but it is recommended to optimize this.
    b. Fluorophore labeled nanobodies will work better and reduce the incubation time required.
5. Wash the samples with PBS (6 x 20 minute washes, 37°C, 50rpm)

Imaging

There are two main imaging approaches when using cleared tissues and organoids: lightsheet microscopy and confocal laser scanning microscopy. Both have their advantages and disadvantages.

Factor	Imaging Modality	
	Confocal Laser Scanning Microscopy	Lightsheet Microscopy
Speed	Can be extremely slow requiring hours to image a single sample due to each pixel of the image having to be imaged sequentially.	Much faster: can image samples in as little as 5-10 minutes due to the lighsheet illuminating a wide swathe of tissue at a time.
Resolution	Extremely high due to high quality optics and focussed illumination point afforded by the laser.	Generally limited (0.5-5µm pixel size) due to lower power objectives and the width of the lightsheet (≈20µm).
Photobleaching	Generally suffers much more due to the slow imaging and the high illumination focussed on a small point.	Not as prone to photobleaching because illumination intensities are lower and more spread out.
Sample mounting	Immersed in mounting media in an imaging dish which provides support.	Immersed in a chamber of mounting media therefore needs glueing to the manipulator and the sample has to be able to support its own weight.

Table 2. Comparison of imaging modalities for cleared tissue

Guidelines, precautions, troubleshooting

Please follow the below table to resolve any problems encountered when using this kit. For any problems not listed or for any further advice please contact our technical support team at technicalhelp@hellobio.com.

Problem	Potential cause	Suggested solutions
Solution A or B contain crystals	The solution was not sufficiently warmed before use	• Incubate solution for longer at 37°C or use a water bath to increase the temperature more rapidly. • Vortex the bottle to redissolve the crystals
Tissue insufficiently cleared	Insufficient clearing time	• Increase the incubation time in both clearing solution A and clearing solution B
	Insufficient clearing solution volume	• Increase the volume of tissue clearing solution A and B alongside the volume of the mounting and storage solution
	Sample contamination with blood	• Perfused tissue is preferred as this removes the strongly colored blood that can interfere with the clearing of highly vascularised tissues.
High autofluorescence	Overfixation	• Reduce the incubation time with 4% PFA
	Insufficient washing	• Increase the number and length of washes
Cleared tissue has turned orange / brown	Oxidation of sample	• Reduce the time between sample collection and processing as much as possible. • Where possible ensure all sample tubes are completely filled to reduce the amount of air.
Poor antibody staining	Low antibody concentration in tissue	• Increase the concentration of primary and secondary antibody • Increase the incubation time for primary and secondary antibody
	Antibody doesn't work	• Check that the antibody works in non-cleared tissue before testing in cleared tissue.
Poor antibody penetration	Poor diffusion of the antibody into the sample	• Increase the incubation time • Reduce the size of the antibody by using nanobodies, Fab fragments or Sc-Fvs. • Use fluorescently labelled primaries to remove the need for secondary antibodies to penetrate the sample.
The size of the sample has grown	Expected result during the protocol	• This is an expected observation during the protocol and will be reversed after using the mounting and storage solution.

Observe safe laboratory practice and consult the safety datasheet. Please see the datasheet on our website for general guidelines, precautions, limitations on the use of the assay kit.

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