Organic solvent based clearing using THF and DBE

The protocol described below is one of certainly several different protocols for clearing tissue. Due to the characteristics of each single tissue it is useful to optimize the protocol regarding to the different incubation times. Too extended incubation in dehydration solution will delete protein based fluorescence like in case of GFP. Please keep the dehydration times as short as possible. But also consider that a less complete dehydration will cause a turbid appearance of the sample. Samples which do not contain any GFP or other fluorescent proteins can be incubated in 100% THF overnight for a perfect clearing result.

Samples stained with Alexa dyes or other chemical dyes are normally not affected at all by the clearing procedure. An exception to this rule is Dil or other dyes binding loosely to lipid structures.

If possible please use dyes with an excitation maximum above 450 nm. Short wavelengths like for instance 405 nm are less suited for imaging of large samples. The shorter a wavelength the more scattered it gets by the tissue. To penetrate large and dense tissue one should use excitation wavelength above 500 nm. Please avoid the usage of DAPI if possible. As a marker for the nuclei TROPO can be used.

Sample preparation

If you run already successful perfusions there is no need to change your protocol referring to the procedure described below.

The correct sample preparation is crucial for a successful clearing and image acquisition of specimens to obtain the protein based fluorescence. Remaining blood in the vascular system of the animal will cause a strong fluorescence outshining most specific fluorescence. The brief fixation of the animal with PFA will help preserving the fluorescence of proteins like GFP. The protocol describes the perfusion and sample preparation of a mouse model. Following reagents are needed:

- pentobarbital (10 mg/kg)
- 40 mL of ice-cold 0.1 M PBS (pH 7.4) Heparin containing 1000 units/mL heparin
- 80 mL of 4% paraformaldehyde (PFA) in ice-cold 0.1 M PBS (pH 7.4)
- ice-cold 0.1 M PBS (pH 7.4)
- 30% sucrose in ice-cold 0.1 M PBS (pH 7.4)

Experimental method

1. Anesthetize the mice.
2. Kill the mice by intraperitoneal injection of pentobarbital (10 mg/kg).
3. Open the abdomen and the thorax with perasternal cut.
4. Do not overexpand the cervix of the mouse. Vessels could be occluded.
5. Place a small cut at the apex of the pericardium to enter the left ventricle.
6. Insert a cannula and place it in front of the aorta. The cannula should not to be placed in front of the pulmonary artery.
7. Locate the cannula with clamp.
8. Open the right atrium with a small cut for leaking buffer.
9. Transcardically perfuse the mice with 40 mL PBS containing heparin, followed by 80 mL 4% PFA in PBS.
10. Remove the organs of interest and rinse them two times in PBS at 4 °C.

Monitoring a successful perfusion

- The liver is beige and no longer dark red.
- The whole tissue including the tail is no longer flexible.
- Body parts not covered by pelt are whitish.
- The lung is not inflated. The perfusion did not enter the pulmonary circulation.

Start with the clearing as soon as possible. After storing the samples for a few days without any further processing the protein based fluorescence will be most likely gone.
Clearing Procedure

Dehydration solution
Tetrahydrofuran anhydrous>=99.9% Prod.# 186562-1L Sigma-Aldrich

Clearing solution
Benzyl Ether 98% Prod.# 108014-1KG Sigma-Aldrich

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Mammary gland, lymph node</th>
<th>Spinal cord, lung, spleen</th>
<th>Brain stem</th>
<th>Brain hemisphere</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% (vol/vol) THF</td>
<td>20 min</td>
<td>30 min</td>
<td>1 h</td>
<td>2 - 3 h</td>
</tr>
<tr>
<td>70% (vol/vol) THF</td>
<td>20 min</td>
<td>30 min</td>
<td>1 h</td>
<td>2 - 3 h</td>
</tr>
<tr>
<td>100% (vol/vol) THF</td>
<td>3 × 20 min</td>
<td>3 × 30 min</td>
<td>2 × 1 h</td>
<td>2 x 2 - 3 h</td>
</tr>
<tr>
<td>DBE</td>
<td>2 x 20 min</td>
<td>2 x 30 min</td>
<td>2 x 1 h</td>
<td>2 x 3 h</td>
</tr>
</tbody>
</table>

While clearing the samples please prevent exposure to light.

Samples not containing GFP or any fluorescent protein

In general samples not containing GFP or other fluorescent proteins can be dehydrated overnight (final dehydration step 100% THF overnight).

Small or very thin samples (e.g.: skin or lymph node) should be embedded in 1% low melting agarose using distilled water. Cut out a small cube (up to 1 cm x 1 cm x 1 cm) containing the sample. Clear the cube as described for brain hemispheres including a last dehydration step in 100% THF overnight.

Samples containing GFP or a fluorescent protein

Extended dehydration will decrease the fluorescence of proteins most widely. For this reason incubate fluorescent protein containing samples as short as possible in dehydration solution referring to table above.

Fluorescent protein containing samples should not be embedded in agarose even if they are pretty small. The extended incubation time in 100% THF overnight will delete all fluorescence. For this reason do clearing on empty agarose cubes as described above. In parallel prepare the small samples using short incubation times in THF. The cleared samples can be placed later on in the cleared agarose cube by cutting a small slit into the cube and inserting the cleared sample.