Chapter 16

X-FaCT: Xenopus-Fast Clearing Technique

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Abstract

Accessibility and imaging of cell compartments in big specimens are crucial for cellular biological research but also a matter of contention. Confocal imaging and tissue clearing on whole organs allow for 3D imaging of cellular structures after being subjected to in-toto immunohistochemistry. Lately, the passive CLARITY technique (PACT) has been adapted to clear and immunolabel large specimens or individual organs of several aquatic species. We recently demonstrated tissue clearing on one-week old tadpole brain (Fini et al., Sci Rep 7:43786, 2017). We here describe a further simplified version with clearing of small tissue samples (thickness inferior to 500 μm)) carried out by immersion in a fructose-based high-refractive index solution (fbHRI). By refining steps of the protocol, we were able to reduce the overall procedure time by two thirds. This offers the advantages of reducing the time of experimentation to a week and minimizes procedure-induced tissue deformations. This protocol can be easily adapted to be performed on thick section. We present an example of immunohistochemistry performed on NF45 *Xenopus laevis* brains with anti-pH 3 (phosphorylated histone H3) antibody used to stain chromatin condensation commonly associated with proliferation.

Key words Tissue clearing, Fructose-based high-refractive index solution, 3D imaging

1 Introduction

There is an exponential increase in the number of studies on neurodevelopmental diseases, which show evidence of embryonic origin, especially during neurogenesis processes. Many molecules in the direct environment of the embryo, mammalian, or non-mammalian can interfere with neurodevelopment, especially by interfering with hormonal signaling. It is therefore essential to be able to precisely study the changes in the proliferation, migration, or maturation of neural cells in the developing brain. The external development of aquatic larvae makes the ontology of their central

nervous system tractable over time. Moreover, these larvae are small and have a natural almost transparent appearance. However, pigmentation and relative structure of different tissues make 3D-imaging challenging.

Recently, tissue clearing methods have been developed in parallel with confocal, biphoton, or light sheet imaging techniques. The CLARITY technique [1, 2] and the passive CLARITY technique, also called PACT [3, 4], were originally developed on rodent organs and human tissues. These clearing strategies permit deep image acquisition of thick 3D objects.

The Tefor Core Facility adapted the PACT technique to clear and immunolabel large specimens or organs of aquatic species. We modified and adapted the CLARITY method for use in aquatic species including fish, such as medaka (*Oryzias latipes*) [5], and zebrafish (*Danio rerio*) [6]. We also transposed this protocol, named Z-PaCT, to NF45 *Xenopus* larvae and brains where we were able to label neurons and oligodendrocytes in tadpole brains [7]. However, one limitation of this technique, besides the image system accessibility, is the time needed to reach sensitivity. Indeed, immuno-labeling using specific antibodies took 2.5–3 weeks for the whole procedure and slightly affected tissue integrity.

Here, we describe a simplified version of this recent protocol where we obtained tissue clearing by simple immersion in a fructose-based high-refractive index solution (fbHRI). By refining numerous steps of the protocol, we were able to reduce the overall procedure time to 1 week instead of 2.5/3 weeks previously. This offers the double advantage of reducing the time of experimentation but also minimizes tissue modification during the procedure.

We here present the use of this protocol and results of immunohistochemistry performed on the brains of NF45 *Xenopus laevis*. The tadpoles were exposed to thyroid hormone, which is known to trigger cell proliferation, for 72 h fixed and stained with anti-pH3 antibody marking chromatin condensation. We used anti-pH3 because it is commonly acknowledged as a proliferation marker. We are confident that this protocol will be useful for in toto immunohistochemistry on both heads and brains but also on thick sections.

2   Materials

2.1   Tadpole Rearing

1. Aquatic system water, chlorine-free.

2.2   Treatment with Thyroid Hormone T₃

1. T_3 stock solution: 10 mM 3,3′,5-Triiodo-L-thyronine sodium salt (T_3) in 30% NaOH) (Sigma Aldrich). The 10 mM stock solution of T^3 is made by adding 100 mg of T^3 powder to 14.86 mL of 30% NaOH (obtained by mixing 4.458 mL of 1 N NaOH with 10.402 mL Milli-Q (ultrapure) water). Vortex

the T_3 stock solution well, aliquot and store at −20 °C. Prevent multiple weighing due to the hygroscopic properties of the powder.

2. Dimethyl sulfoxide (DMSO) (Carl Roth).

3. 1 mL transfer pipettes (Samco Scientific).

4. 50 mL polypropylene conical tube (Corning).

5. 6-well plate TPP (Thermo Fisher Scientific).

6. Incubator at 23 °C protected from light.

2.3 Brain/Head Preparation

1. Dumont tweezers, #2 straight (World Precision Instruments).

2. 60 × 15 mm Petri dishes (Greiner Bio One International).

3. 1× Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4.

4. 2× Phosphate-buffered saline (PBS): 274 mM NaCl, 5.4 mM KCl, 20 mM Na_2HPO_4, 3.52 mM KH_2PO_4, pH 7.4.

5. PBST: 1× PBS, 0.01% Tween® 20, pH 7.4.

6. Ethyl 3-aminobenzoate methanesulfonate salt (MS222) (Sigma-Aldrich).

7. 8% paraformaldehyde in distilled water prepared from paraformaldehyde powder (Sigma Aldrich). Prepare aliquots of 8% PFA in H_2O (dissolved at 60 °C and stored at −20 °C). For 4% PFA dilute in 2× PBST. Another option is to buy ready-to-use 16% formaldehyde (w/v), methanol-free capsules (Thermo Fisher Scientific).

8. Fixing solution: 4% paraformaldehyde (PFA) in PBST.

9. Storage solution: 0.5% PFA in PBST with 0.05% sodium azide.

2.4 Depigmentation

1. Pre-incubation solution: 0.5× SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.2), 0.1% Tween® 20.

2. Depigmentation solution: 5% formamide, 0.5× SSC, 3% H_2O_2.

2.5 Immunolabeling

1. 2 mL glass vials (Carl Roth).

2. Blocking solution: 10% normal goat serum (Antibodies Incorporated), 10% DMSO, 1% Triton X-100 in PBST.

3. Primary antibody: e.g., mouse anti-pH3, 1/200 (Millipore).

4. Secondary antibody: e.g., goat anti-mouse Alexa fluor-555, 1/200 (Thermo Fisher Scientific, Invitrogen).

5. Staining solution: 2% normal goat serum, 10% DMSO, 0.1%Triton X-100, 0.05% sodium azide, in PBST.

6. DiI (DiIC18(3)) (Thermo Fisher Scientific, Invitrogen). Prepare 1 mM DiI stock solution by dissolving DiI powder in DMSO.

7. DiD (DiIC18(5)) (Thermo Fisher Scientific, Invitrogen). Prepare 1 mM DiI stock solution by dissolving DiI powder in DMSO.

2.6 Tissue Clearing in Fructose–Based High–Refractive Index Solution (fbHRI)

1. Refractometer (e.g., KRUSS Optronic DR 301-95).

2. D(−)-fructose (Carl Roth).

3. Fructose-based high-refractive index solution (fbHRI):

This clearing solution is obtained by mixing three solutions prepared separately in advance:

(a) fbHRI Diluent: 0.2× PBS, 0.002% sodium azide.

(b) 90% fructose solution (RI = 1.457 (F1457)). In a beaker, dissolve 800 g of fructose in 300 mL fbHRI diluent. Cover the solution to avoid evaporation and stir with a strong magnetic stirrer. Make up the volume to 800 mL with fbHRI diluent. Measure RI and adjust the fructose solution to an RI of 1.457 with fbHRI diluent.

(c) 85% DMSO solution (RI = 1.457 (DMSO1457)). Adjust the DMSO solution to an RI of 1.457 with fbHRI diluent.

Mix F1457 and DMSO1457 in the ratio of 4/1 (v/v). Adjust RI with 100% fructose to raise RI or diluent to lower RI. This solution can be stored for 6 months at 4 °C.

2.7 Mounting and Imaging

1. Low-melting point agarose (e.g., Sigma-Aldrich).

2. Fluorescent microscope, for example Leica TCS SP8 laser scanning microscope (Leica Microsystems, model: Leica TCS SP8) with two photomultipliers (PMT) and equipped with a Leica HC FLUOTAR L 25×/1.00 IMM motCorr objective (Leica Microsystems).

3   Methods

3.1 Tadpole Rearing

Xenopus laevis embryos and tadpoles are reared at 20 °C in chlorine-free water (photoperiod = 12 h/12 h light/dark), in groups of 70 individuals per 15 L tank. All procedures are performed in accordance with European Union Directive 2010/63/EU.

3.2 Treatment with Thyroid Hormone T_3

1. Make a 10^{-4} M intermediate solution of T^3 fresh every day by adding 5 μL of 10 mM stock solution of T^3 to 495 μL of chlorine-free water (*see* **Note 1**).

2. Add 2.5 μL of 10^{-4} M T^3 to 50 mL of chlorine-free water to create a final 5 nM T^3 solution. Vortex the solution thoroughly (*see* **Note 2**).

3. Using a 1 mL transfer pipette with open section cut, transfer fifteen st.45 tadpoles into one well of a 6-well plate containing 8 mL chlorine-free water. In this standard assay, 15 tadpoles are used per exposure group per replicate. Rinse every 6-well plate with chlorine-free aquarium water before use.

4. Empty the liquid in the 6 wells using transfer pipette. Add 8 mL of 5 nM T^3 test solution previously prepared in Subheading 3.2, **step 2** into the well. Place the 6-well plate into a dark incubator at 23 °C for the next 72 h.

5. Renew the solutions every day at the same time (±1 h). Remove any dead tadpoles (*see* **Note 3**).

3.3 Brain/Head Preparation

1. Prepare the anesthetic solution: 1 g/L MS222 solution (1 g MS222, 1 g sodium bicarbonate in 1 L chlorine-free water). Adjust the pH between 7.2 and 7.8. Protect from light, store for 3 weeks (*see* **Note 4**).

2. Under the fume hood, prepare fresh 4% PFA fixing solution from thawed 8% PFA (H$_2$O) in 2× PBST (*see* Subheading 2.3, **item 7**). Prepare 40 mL of fixing solution 4% PFA in PBST in a 50 mL conical tube for 5 tadpoles. Keep the tubes at 4 °C for 1 week.

3. Kill the specimens in 1 g/L MS222 solution in accordance with local regulations. Usually 2–3 min are enough for one-week-old tadpoles.

4. Fix tadpoles overnight at 4 °C in fresh fixing solution or at room temperature for 3 h.

5. If needed, store the sample in storage solution at 4 °C until further use.

6. Brain dissection: place tadpoles in 1× PBS under fume hood. Dissect brains in the liquid. Remove skin, move a small forceps under brain tissue, and cut spinal cord (see movie dissection accessible at URL www).

3.4 Depigmentation

(Optional for ventral acquisition of brain tissues)

1. Wash in 1× PBST samples at least 1 day before starting the depigmentation needed.

2. Pour 10 mL of pre-incubation solution in a 60 mm Petri dish and transfer the fixed samples to the Petri dish with 1 mL transfer pipette.

3. Incubate at room temperature for 1 h.

4. Prepare the depigmentation solution under the fume hood.

5. Under the fume hood, remove the samples with some liquid and place them in the lid of the Petri dish. Remove all of the pre-incubation solution from the dish and replace it with

depigmentation solution. Return the samples to the dish. Keep the Petri dish uncovered and under light to accelerate the depigmentation process.

6. Check the progress of the depigmentation every 10 min.

7. Once the depigmentation is complete (*see* Fig. 1), under a fume hood rinse twice in PBST. The standard depigmentation time is 90–120 min for intact *Xenopus* NF45 larvae and 45 min for dissected brains.

8. Rinse samples overnight or for a minimum of 4 h in several changes of PBST.
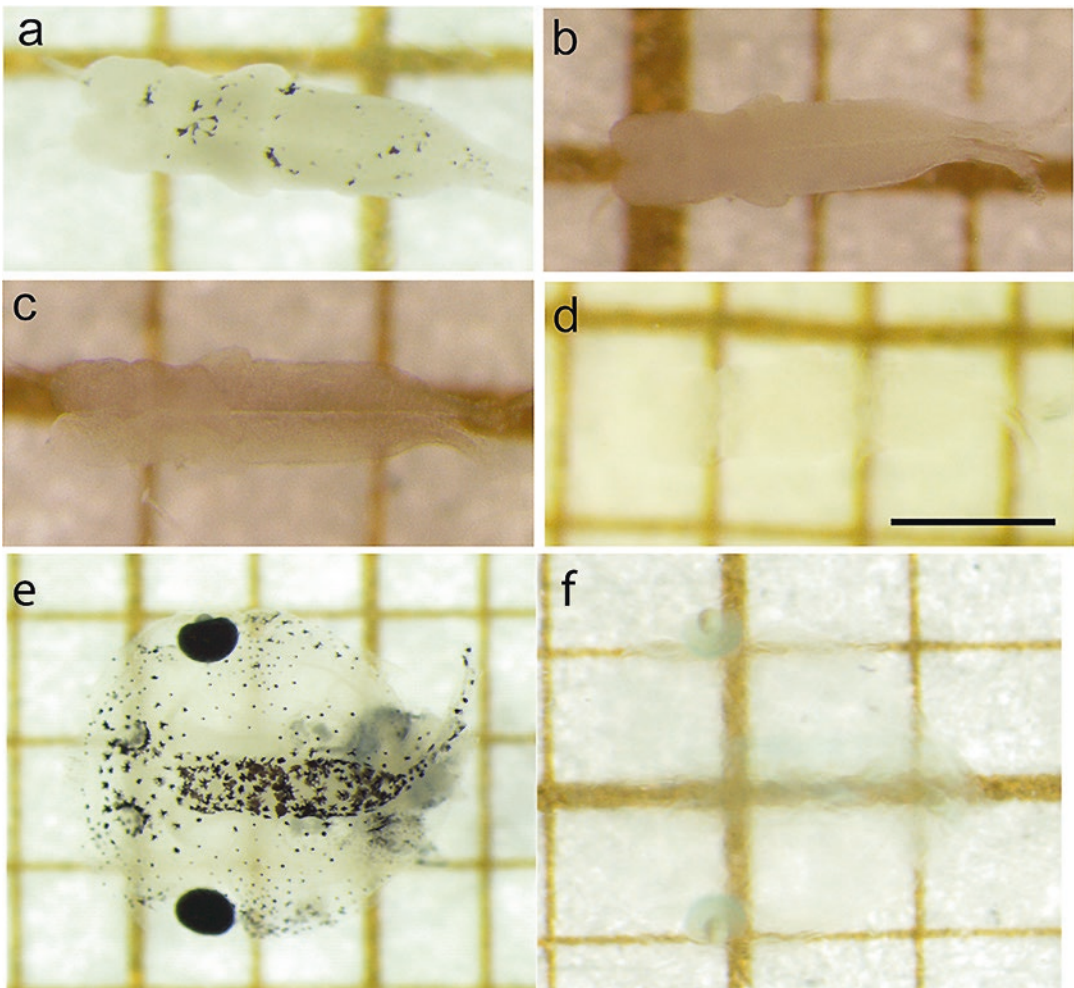
Fig. 1 Tissue clearing of Xenopus brains stage NF45. Final clearing of NF45 Xenopus laevis brains and heads after passive clearing by homogenizing the refractive index in fructose based high refractive index medium (fbHRI). (**a**) Dissected NF45 Xenopus laevis brain in PBS after PFA fixation (Subheading 3.3). (**b**) Removal of superficial pigments by H_2O_2 bleaching (Subheading 3.4). (**c**) After incubation in 50% fbHRI the opacity is significantly reduced (Subheading 3.6, **step 2**). (**d**) The tissue clearing process is finished after incubation in 100% fbHRI (Subheading 3.6, **step 4**) (**e**) NF45 *Xenopus laevis* head in PBS after PFA fixation (**f**) same head after depigmentation. Scale bar = 500 μm. All brains/heads are anterior to the left

3.5 Fluorescent Staining	1. Transfer the samples to 2 mL glass vials using a truncated transfer pipette.

1. Transfer the samples to 2 mL glass vials using a truncated transfer pipette.
2. Remove the liquid and add 1 mL of blocking solution.
3. Incubate overnight at 4 °C or for 2–4 h at room temperature.
4. Remove the blocking solution and add 600 μL of staining solution.
5. Add primary antibody to obtain the working dilution.
6. Incubate at room temperature for 2–3 days, with gentle shaking.
7. Rinse 3 times, for 20 min each, in PBST.
8. Remove all the PBST and add 600 μL of staining solution per tube.
9. Add fluorescent secondary antibody.
10. Incubate at room temperature for 2 days, with gentle shaking.
11. Rinse for 1 day in several changes of PBST.
12. Optional: Incubate the samples for 2 days in 600 μL of staining solution containing 1 μM DiI/DiD.
13. Rinse the DiI-stained sample in PBST for a minimum of 3 h.

3.6 Tissue Clearing

1. Prepare fbHRI (see 2.6 point 3), make sure to adjust the fbHRI to the desired refractive index.
2. Incubate the samples in 50% fbHRI/50% (v/v) diluent for at least 6 h (Fig. 1).
3. Remove the solution and replace by fbHRI.
4. Incubate for at least 12 h (Fig. 1).

3.7 Mounting and Imaging

Mounting strategies depend on the microscopy technique one may want to apply. Confocal microscopy, multiphoton microscopy, or light sheet microscopy are well suited techniques to image cleared samples. An example of a mounting method is given here. It is optimized for the use of an upright confocal microscope equipped with a dipping lens RI-corrected objective (Leica 15507703).

1. Place your sample in a 60 mm petri dish and remove as much liquid as possible.
2. Pipet 2–5 μL of 1% agarose (50 °C) and use it to glue the sample to the petri dish.
3. Allow the agarose to solidify at 4 °C for 2 min.
4. Fill the petri dish with fbHRI adjusted to the right RI.
5. Dip objective into the fbHRI and capture fluorescence signals by exciting the fluorophores with the appropriate laser wavelength. Tilescan imaging followed by mosaic stitching is required to capture images of the whole brain.

3.8 Visualization of 3D Data Sets (Supplementary Material)

1. Translation movies (connected to Fig. 2a, b) are accessible at the link URL under the names Movies 1, 2, and 3. These movies are showing tapdole brains, which were exposed for 72 h to T_3, processed using X-FaCT with pH3. The translation movies constitute a direct conversion of the raw data into mp4 movie file format. In this process, using a combination of fiji [8] and libav (https://libav.org/), each slice (z-step) of the raw-data stack is turned into a frame of the movie turning the z-dimension of the stack into the temporal domain of the movie. Lossy compression (×246-driven h246-compression) provides significant data reduction while keeping the image information largely intact. The original 12 bit histogram of the original data is linearly converted to the necessary 3×8 bit (RGB) without application of any contrast enhancement or noise reduction. This allows insight into a multi-GB-dataset while dealing with only tens of MBs.

2. Rotation movies (connected to Fig. 2c, d) are accessible at link www. Under the names Movies 4 and 5. The rotation movies can be generated using amira 6.0.1 (FEI, SAS) on a Dell T630 running Ubuntu 16.04.

Since in the original data of the pH3-channel we found a low but distinct amount of signal bleed-through from the DiD stain, we subtracted a fraction of the DiD-channel from the pH3-channel ([pH3]-([DiD]/5). The division coefficient thereby was determined empirically for selectively removing the background signal. This process allows subsequent enhancing of the contrast of the pH3-signal by global linear histogram stretching (GLHS), which results in the pH3-positive cells appearing brighter, which again
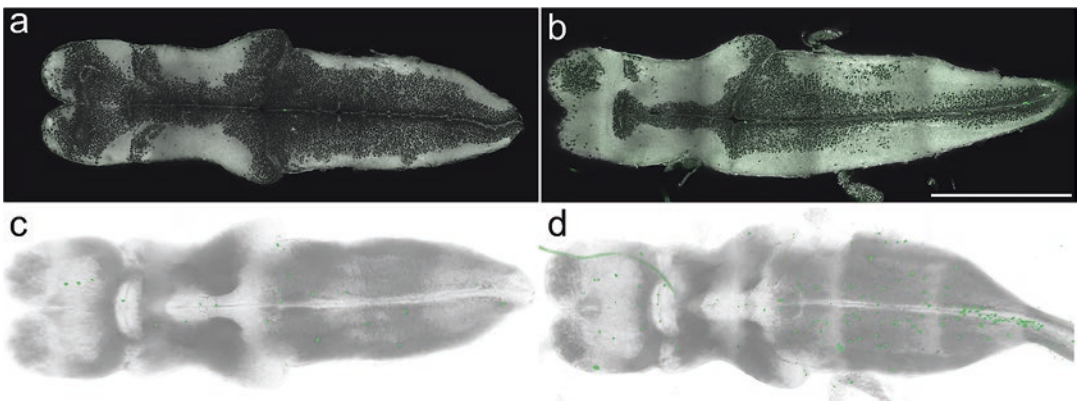
Fig. 2 NF45 Xenopus laevis whole-brain immunolabeling and imaging. Single confocal plane of a NF45 control brain (**a**) or treated with 5 nM T_3 (**b**) stained with DiD (gray), immunolabeled for pH 3 (green). The visualisation of the whole dataset is provided by two translation movies, control brain (Movie 2) or T3 treated (Movie 3) provided at the URL www. Scale bar = 500 μm. Ventral 3D views from rotation movies of control brain (**c** and Movie 4) or T3 treated (**d** and Movie 5) accessible at the URL www. All brains are anterior to the left

facilitates their detection in the 3D-visualization. To the reference channels (DiD) we applied only GLHS without any other pretreatment. Due to slightly different acquisition settings between the specimens the GLHS settings were optimized for each dataset individually.

[[untreated: pH3: 150–1300, DiD: 0–3500; treated: pH3: 150–2000, DiD: 0–2800. All values on the basis of a 12 bit histogram (0–4095)]].

4 Notes

1. Pay attention that neither precipitate nor crystals are present in the T_3 stock.

2. Be careful to rinse every 50 mL plastic tube with dechlorinated animal system water before use.

3. If the mortality exceeds 20% in a well, stop and repeat the experiment.

4. Consider making a stock solution at 4 g/L.

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