Ex vivo staining of embryos (mouse) with phospho-tungstic acid for soft tissue contrast in micro-CT imaging

Method note

MCT-007

1. Introduction

Embryology and developmental biology studies require high resolution 3d imaging of embryos, such as of the mouse. However without contrast agent staining, very little soft tissue detail is visible. Here a soft tissue contrast agent, phosphotungstic acid (PTA) is used to stain mouse embryos for micro-CT scanning.

PTA has the chemical formula $H_3PW_{12}O_{40}$ (see the sidebar from the Wikipedia entry, figure 1 [ref. 1]) Tungsten is a high atomic number (74) transition metal and thus confers strong x-ray contrast when attached to biological tissue. PTA binds to fibrin and collagen, proteins that are ubiquitous in connective tissue throughout all animal organs. This affinity, combined with composition and micro-architectural differences between biological tissue, mean that in practice PTA shows a wide range of densities in tissue corresponding to different tissue types, and is thus a very useful contrast agent.

Further, as this note will demonstrate, staining in a solution of PTA in ethanol (sometimes called "EPTA") is very straightforward requiring little by way of materials, equipment or skill.

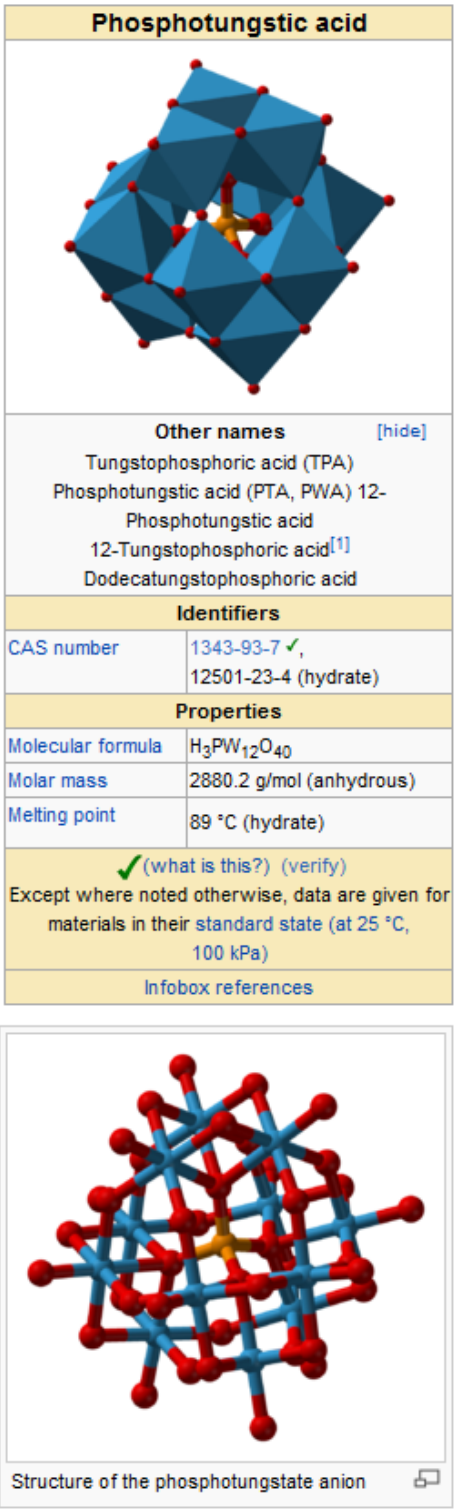


Figure 1 (right): panel of data from Wikipedia on phosphotungstic acid (PTA).

PTA is also widely used as a stain for both microscope histology, and electron microscopy. Staining of embryos of various vertebrate and invertebrate animals by PTA and other stains such as iodine has been described by Metscher *et al.* [ref 2].

2. Method

Embryos of mice (figure 2) are stored in buffered formalin (buffered formol saline) prior to EPTA staining and micro-CT imaging.

Figure 2. Embryo of a mouse (day 15) taken from buffered formalin storage.

(a) A solution of PTA in ethanol ("EPTA") is prepared. Approximately 1 ml of phosphotungstic acid hydrate, a dry powder (Alfa Aesar – [ref. 2]) is mixed into 100 ml of 70% ethanol, in which the PTA powder dissolves rapidly and completely. That is, about a 1% solution by volume. Note that this PTA hydrate powder should be stored in a desiccator chamber to maintain dryness.

(b) The phosphate buffer in formalin can oppose the perfusion of PTA through a tissue sample. Therefore where samples have stored in buffered formalin, an important first step is washing the sample in tap water. This can be achieved by placing the sample, such as an embryo, in a beaker in a sink with tap water running into it, for several hours.

(c) Following this, the next step is serial dehydration in ethanol:

- A few hours or overnight in 30% ethanol
- A few hours or overnight in 50% ethanol
- A few hours or overnight in 70% ethanol

(d) After this serial dehydration, individual mouse embryos are immersed in a few ml of this PTA-ethanol (EPTA) solution in small sealed plastic flat-bottomed vials. They are left in the stain from 2-4 days. Note that larger soft tissue samples require longer immersion in the PTA-ethanol solution, up to 2 weeks.

(e) Following staining, the embryos are removed from the EPTA onto paper tissue and superficially dried for a few seconds. Then they are wrapped in thin strips of soft paper tissue, and mounted for micro-CT imaging, for instance in the SkyScan plastic sample holder tube (figure 3; inner diameter 10mm). Once the paper-wrapped embryos are in the plastic tube, they are wetted with a few drops of tap water (or 0.9% buffered physiological saline) moistening the paper around the embryo to prevent drying during the scan. Alternatively plastic film (non-PVC) or parafilm can be used, either alone or with additional outer wrapping with moistened paper tissue.

Note that it is also possible to stain biological tissue in a solution of PTA in water rather than ethanol. If available, a lid can be placed on the micro-CT sample holder tube.

Figure 3. SkyScan plastic tube sample holder set – standard inner tube diameters are 6, 10, 15 and 20mm.

If the vertical plasic tube sample holder is not being used, then it is advisable if possible for the plastic and/or paper tissue wrapped embryo to be placed in a small eppendorf plastic tube and the tube closed, to exclude air exchange with the outside. Other tube mounting solutions are also possible. It is very important to prevent any dehydration of the embryo during the scan.

Embryos are scanned in the SkyScan 1172 scanner with the following parameters:

- 60 kV, 150 uA
- 0.5mm Al filter
- Medium 2k resolution level
- Pixel size in the range 4-8 microns depending on size of embryo and on whether a single scan rotation of the whole sample was preferred or multi-part oversize imaging.
- 0.2-0.4 degree rotation step
- 360 degree scan
- 2-4 x frame averaging

Suggested reconstruction parameters in SkyScan NRecon:

- Smoothing: Gaussian (advanced tab) 1-4 depending on visible signal / noise
- Ring artefact reduction: 4-10 (minimum needed to remove most rings)
- Beam hardening correction 40%

- Advanced tab: defect pixel mask at 3-8% (check for artefacts at low values)
- Contrast limits: minimum zero, maximum, 10-20% above maximum end of density histogram
- Set ROI around embryo cross-section to reduce volume of reconstructed files

3. Results

Reconstructed cross-sections from the stained embryo after both 1 day and 4 days of staining showed strong differential staining of soft tissues. Figure 4 below shows sectional slices revealing considerable soft tissue and organ detail (see also figures 6-7). The liver takes up the PTA stain particularly strongly, but the lung also stained with a high density showing lobular and alveolar tissue detail. PTA is knows to stain muscle fibber bundle structure effectively and this was evident in the embryo heart.

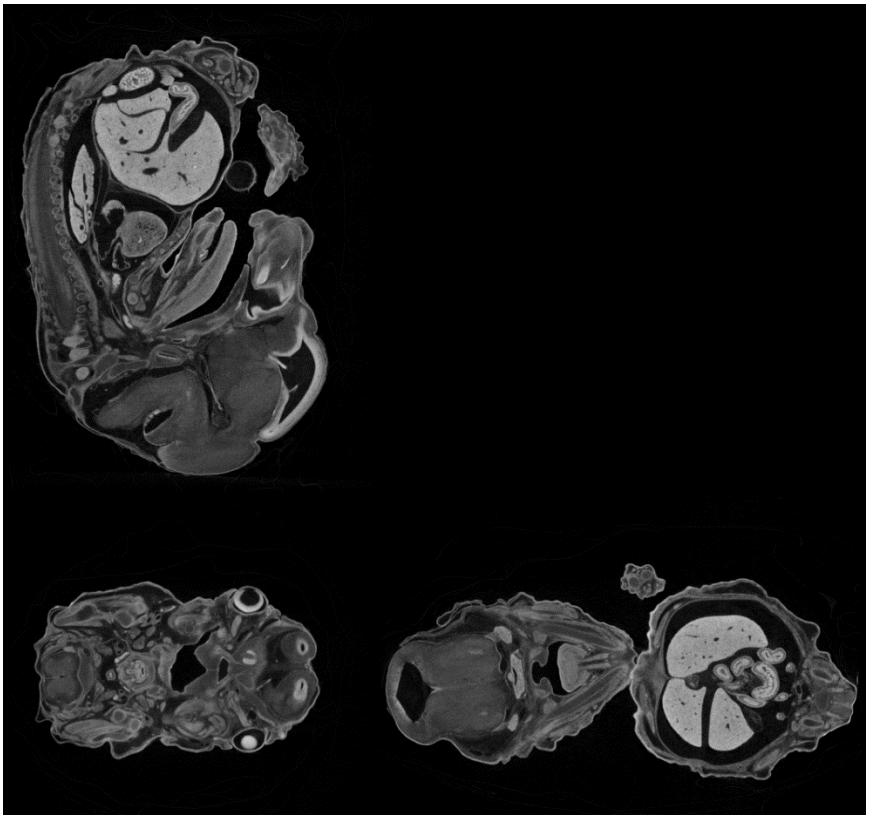


Figure 4. Reconstructed orthogonal cross-sections from a mouse embryo stained for 4 days in EPTA, imaged at 4 micron voxel size in the SkyScan 1172.

Comparison of EPTA stained with unstained embryos confirms the necessity of staining for micro-CT imaging of soft tissue and organ anatomy (figure 5, below).

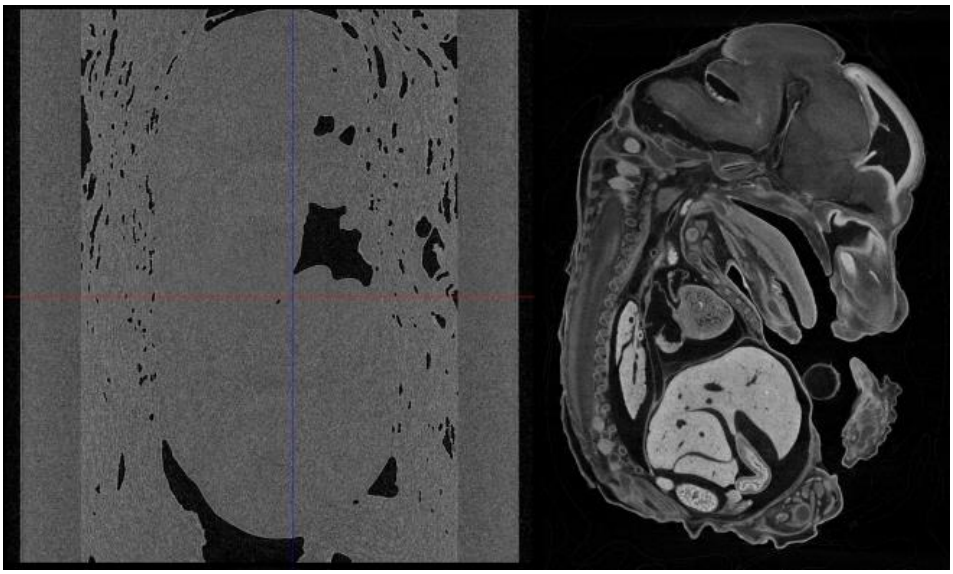


Figure 5. Micro-CT sections of an unstained embryo (left) and an embryo stained for 4 days in EPTA (right).

4. Conclusions

Phosphotungstic acid in an ethanol solution is confirmed as an effective *ex vivo* stain or contrast agent for soft tissues and organs in the mouse embryo. This has been confirmed in other soft tissue samples such as a small fish (figure 7). The staining method is straightforward. However a small amount of shrinkage from dehydration can occur during this process. EPTA staining allows detailed examination of the internal soft tissue anatomy of the mouse and other animal embryos and soft tissue samples. Larger soft tissue samples can be stained by this method using longer immersion times of up to several weeks.

5. References

1. http://en.wikipedia.org/wiki/Phosphotungstic_acid

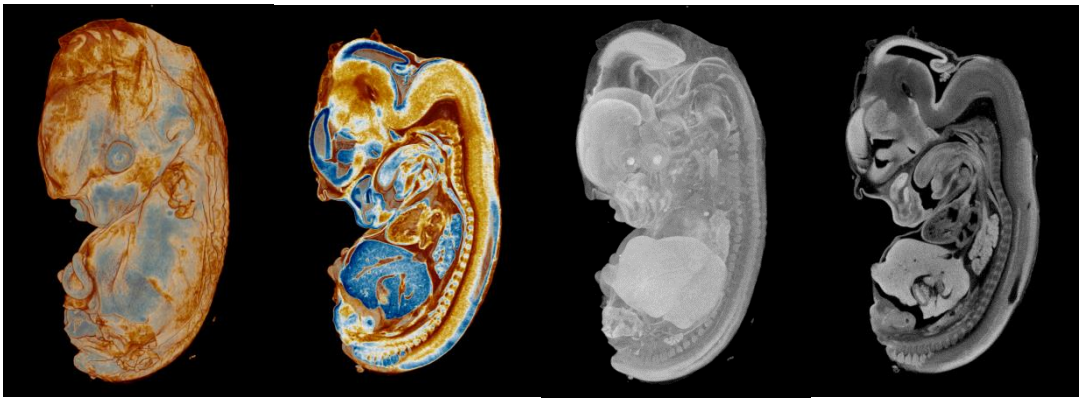
2. Brian D Metscher, MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissue. *BMC Physiology* 2009, 9:11
   Online free pdf of this paper:
   http://www.biomedcentral.com/content/pdf/1472-6793-9-11.pdf

3. http://www.alfa.com/en/GP100W.pgm?DSSTK=40116

4. Lendrum AC, Fraser DS, Slidders W, Henderson R (1962) Studies on the character and staining of fibrin. Journal of Clinical Pathology 15(5): 401-413.

5. Vegesna AK, Chuang KY, Besetty R, Phillips SJ, Braverman AS, Barbe MF, ... & Miller LS (2012) Circular smooth muscle contributes to esophageal shortening during peristalsis. World Journal of Gastroenterology 18(32): 4317.

6. Vegesna AK, Sloan JA, Singh B, Phillips SJ, Braverman AS, Barbe MF, ... & Miller, LS (2013) Characterization of the distal esophagus high-pressure zone with manometry, ultrasound and micro-computed tomography. Neurogastroenterology & Motility 25(1): 53-e6.



(a) (b) (c) (d)

Figure 6. Volume rendered model of the PTA stained embryo, showing the embryo surface (a) and a cut section (b). MIP images of the same stained embryo, showing the whole embryo (c) and a thin sagittal slice (d).

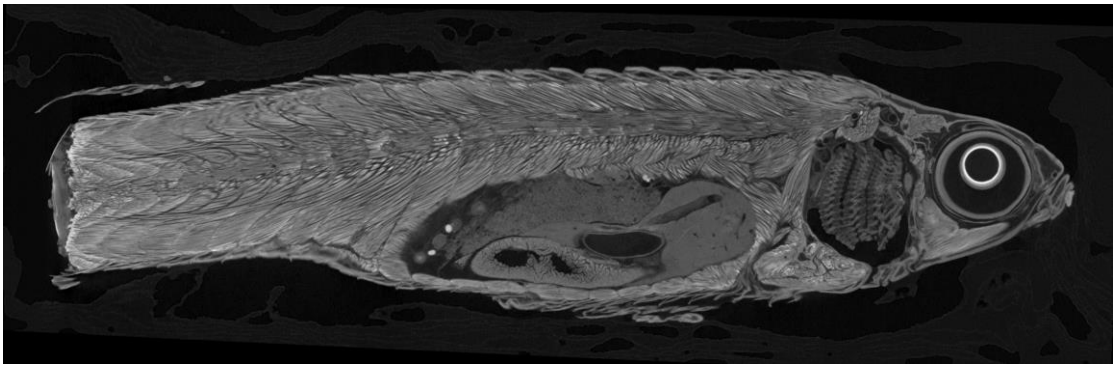


Figure 7. A longitudinal sagittal slice through a zebrafish, scanned by microCT (SkyScan1272) after PTA staining for about 10 days, by the method described in this note.