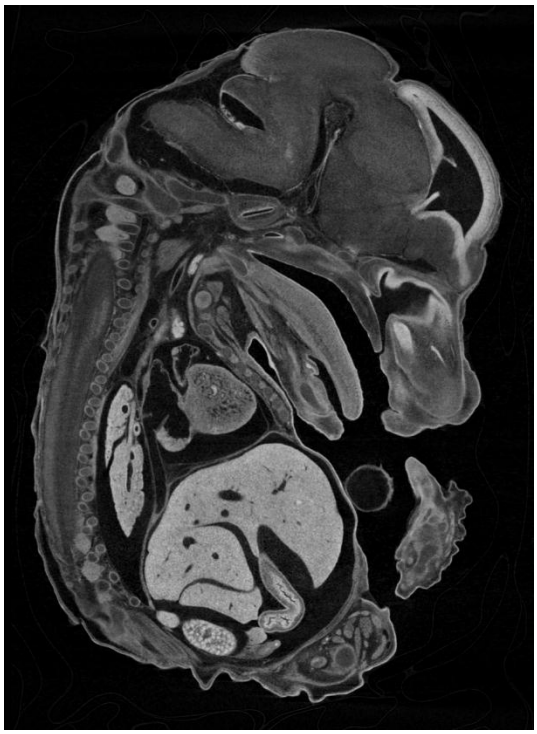
SKYSCAN
X-RAY MICROTOMOGRAPHY
X-RAY NANOTOMOGRAPHY
IN-VIVO SMALL ANIMAL IMAGING

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



Application note

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.

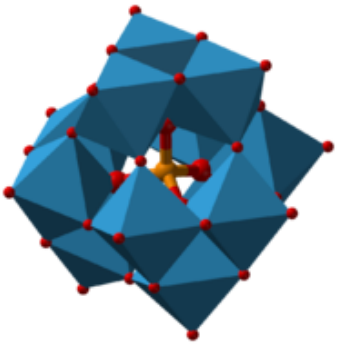
PTA has the chemical formula $H_3PW_{12}O_{40}$ (see the sidebar from the Wikipedia entry, figure 1 [ref. 1]) Tungsten is a high atomic number (74) transition metal and thus confers strong x-ray contrast when attached to biological tissue. PTA binds to fibrin and collagen, proteins that are ubiquitous 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.

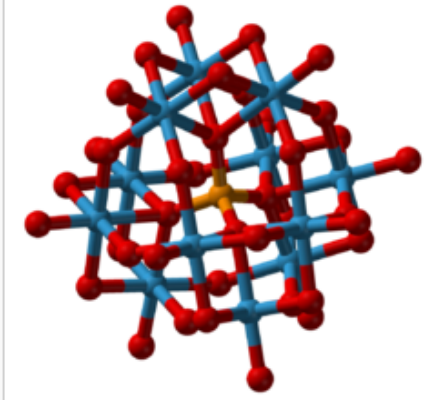
PTA is also widely used as a stain for both microscope histology, and electron microscopy.

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].

Phosphotungstic acid



Other names [hide]
Tungstophosphoric acid (TPA) Phosphotungstic acid (PTA, PWA) 12-Phosphotungstic acid 12-Tungstophosphoric acid[1] Dodecatungstophosphoric acid
Identifiers
CAS number: 1343-93-7 ✓, 12501-23-4 (hydrate)
Properties
Molecular formula: $H_3PW_{12}O_{40}$
Molar mass: 2880.2 g/mol (anhydrous)
Melting point: 89 °C (hydrate)
✓(what is this?) (verify) Except where noted otherwise, data are given for materials in their standard state (at 25 °C, 100 kPa)
Infobox references



Structure of the phosphotungstate anion

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

2. Method

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

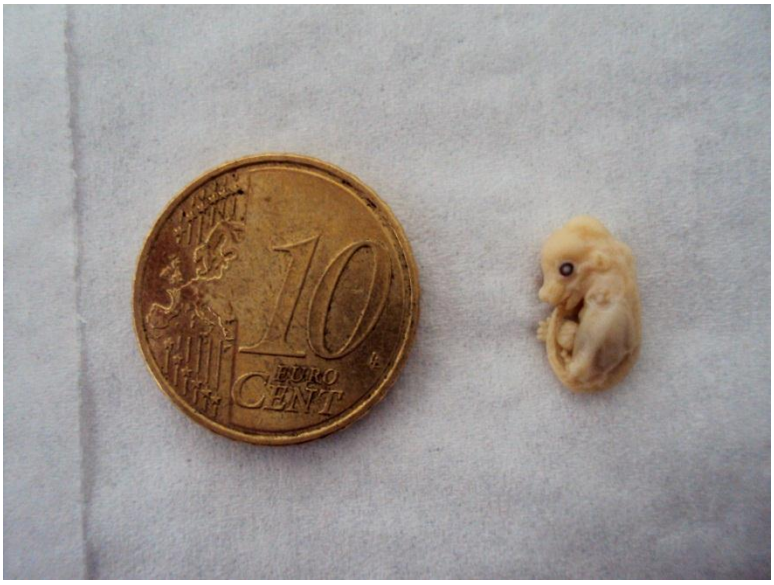


Figure 2. Embryo of a mouse (day 15) taken from buffered formalin storage.

A solution of PTA in ethanol ("EPTA") is prepared. Approximately 5 ml of phosphotungstic acid hydrate, a dry powder (Alfa Aesar – [ref. 2]) is mixed into 200 ml of 70% ethanol, in which the PTA powder dissolves rapidly and completely.

Note that this PTA hydrate powder should be stored in a desiccator chamber to maintain dryness.

Placing embryos in alcohol based EPTA direct from buffered formalin storage can result in some tissue cracking due to rapid dehydration. Therefore the first 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

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 1-4 days.

Then 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 in a plastic sample tube (inner diameter 10mm). Once the paper-wrapped embryos are in the plastic tube, they are wetted with a few drops of tap water moistening the paper around the embryo to prevent drying during the scan. If available, a lid can be placed on the sample tube.

Embryos were 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.
- 0.3-0.5 degree rotation step
- 360 degree scan
- 2-4 x frame averaging

Reconstruction parameters in SkyScan NRecon (1.6.4) were:

- Smoothing: Gaussian (choose in 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, about 20% above maximum end of density histogram (set to log y scale by double-click on the histogram window for easier view of top end of the distribution)
- Where appropriate set ROI around embryo crossection to reduce volume of reconstructed files

3. Results

Reconstructed crossections from the stained embryo after both 1 day and 4 days of staining showed strong differential staining of soft tissues. Figure 3 below shows sectional slices revealing considerable soft tissue and organ detail (see also figures 5-6). 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.

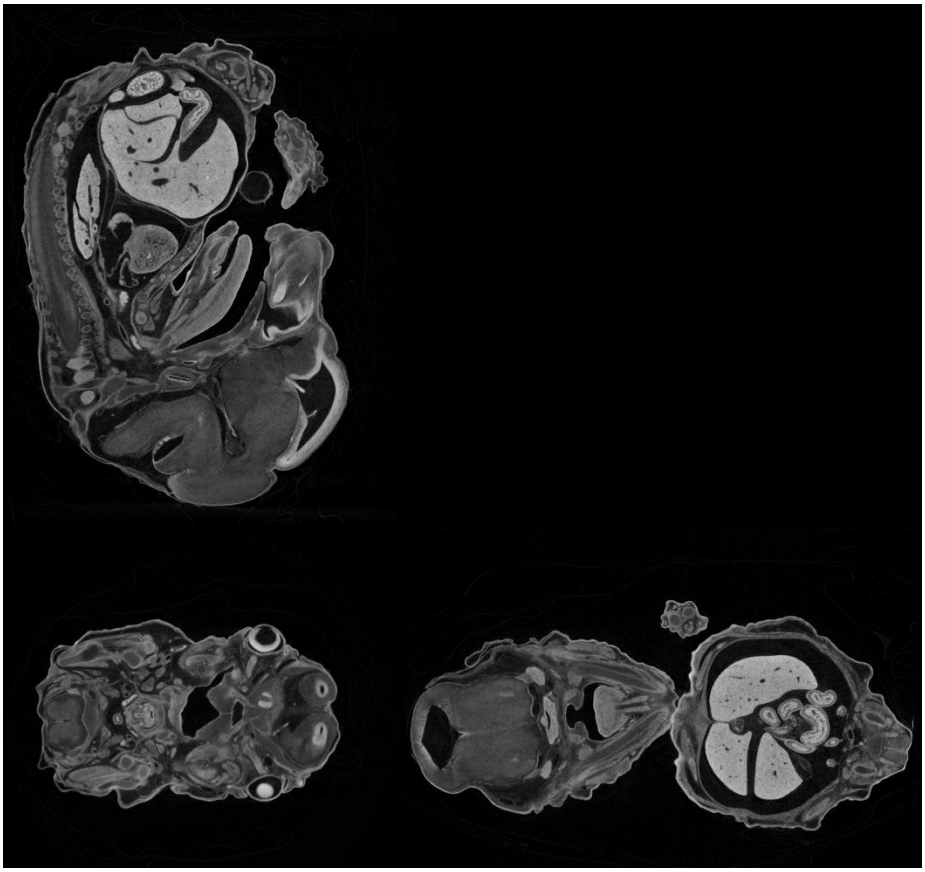


Figure 3. Reconstructed orthogonal crossections from a mouse embryo stained for 4 days in EPTA, imaged at 4 micron voxel size in the SkyScan 1172.

Comparison of EPTA stained with unstained embryos confirms the necessity of staining for micro-CT imaging of soft tissue and organ anatomy (figure 4, below).

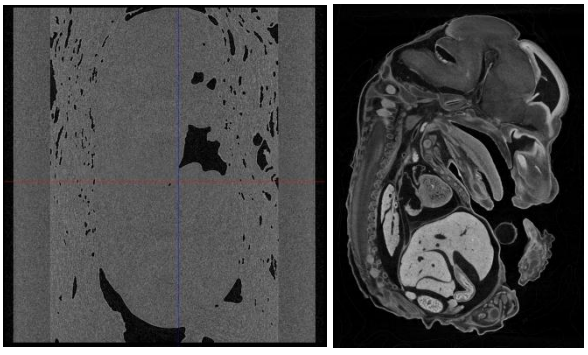


Figure 4. Micro-CT sections of an unstained embryo (left) and an embryo stained for 4 days in EPTA (right).

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. 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. The perfusion of EPTA through tissues was thorough even after 1 day, suggesting that larger embryos and soft tissue samples could also be stained by this method.

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

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

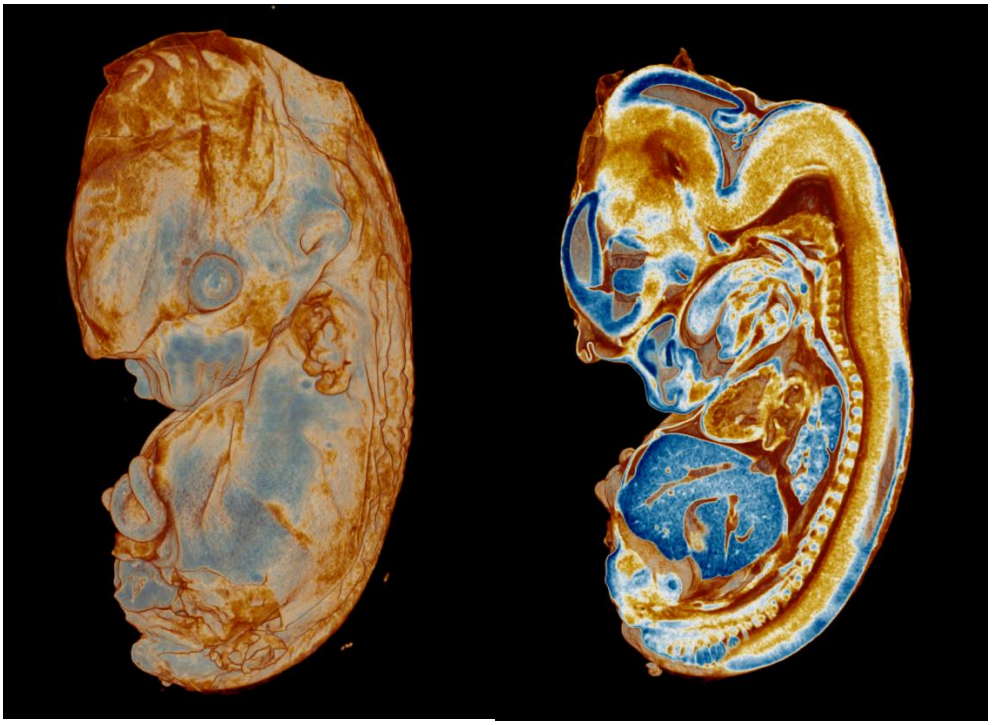


Figure 5. Volume rendered model of the EPTA stained embryo, showing the embryo surface (left) and a cut section (right).

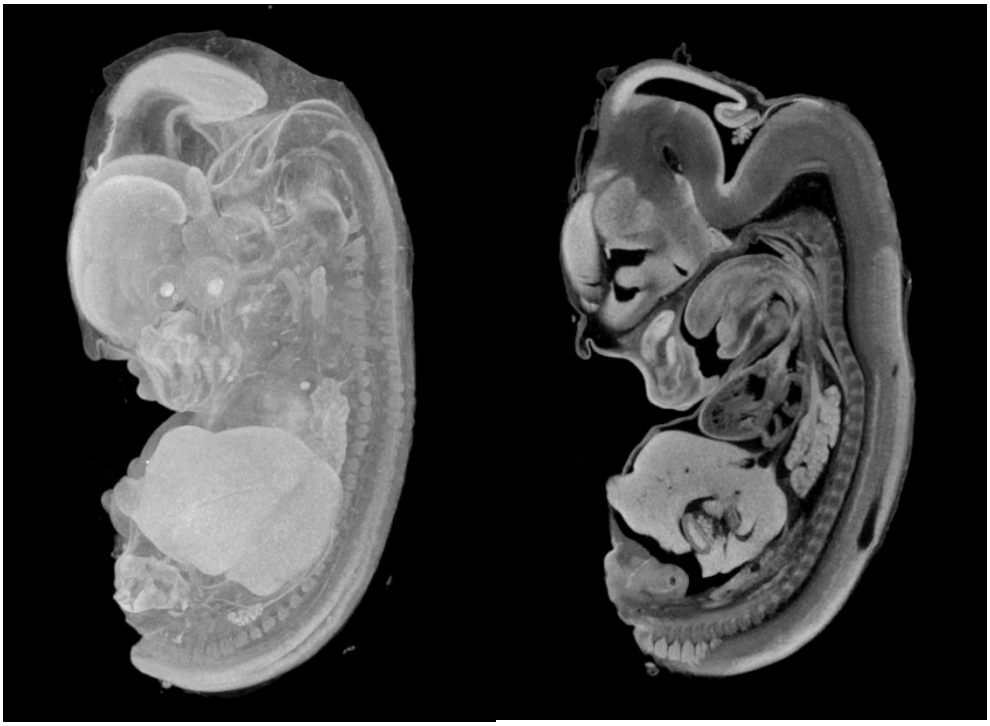


Figure 6. MIP images of the EPTA stained embryo, showing the whole embryo (left) and a thin sagittal slice (right).