Visualisation of developmental ossification using trace element mapping

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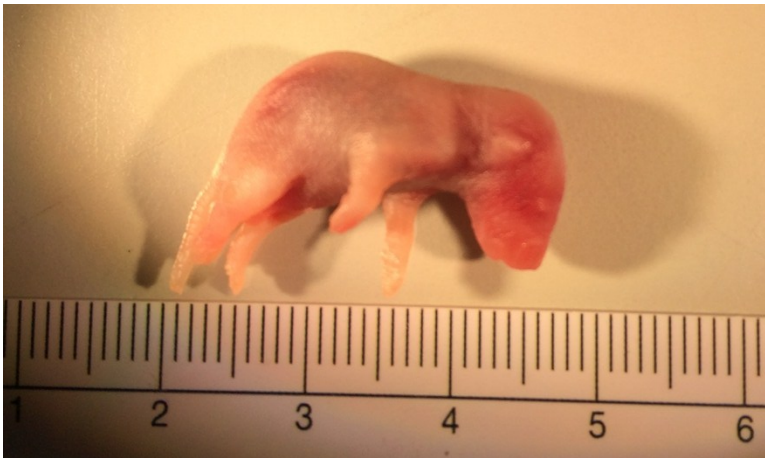
1. Specimens

Specimens consisted of frozen 1-day old mice (*Mus musculus*) purchased from the Monkifield



Nutrition distributor (animal feed company; fig. S1). The forelimb was removed and skinned to

expose the bone and cartilage using a sterile scalpel and a dissecting scope. They were then freeze-dried to prevent rotting while scanning at the synchrotron.

Figure S1: 1-day old mouse used in experiment.

The fossil fish (*Knightia eoceana*) was collected from Fossil Lake basin of the 50 million year old Green River Formation of Wyoming, USA (fig. S2). The sample was analysed as it was extracted from the site with no further preparation, washing, or application of glues or preservatives. Both specimens are housed at the University of Manchester.

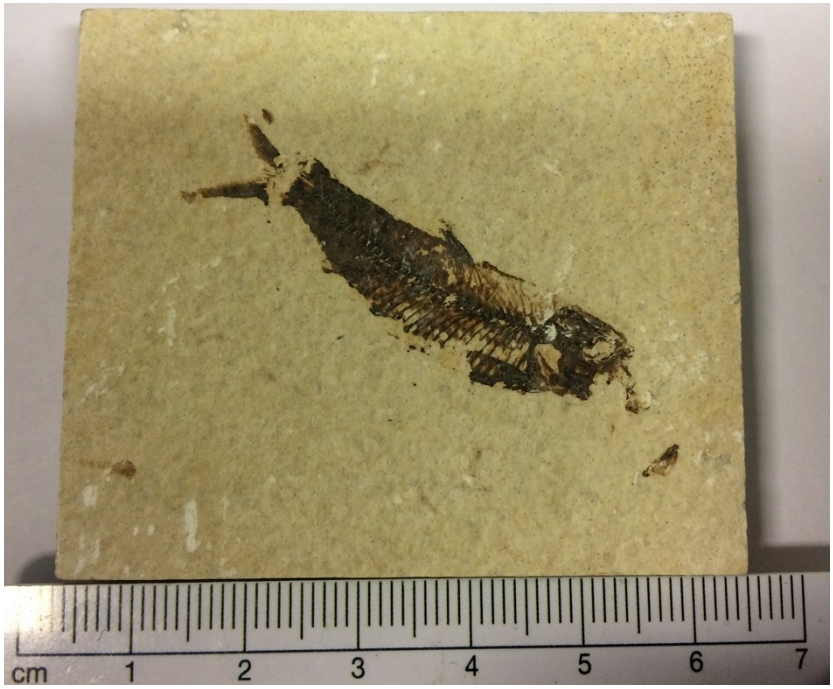


Figure S2: 50 million year old green river fish, *Knightia eoceana* (UM868).

2. Synchrotron XRF Low-Z maps *M. musculus*

P is distributed only within the ossified tissues of the phalanges and upper arm bones, though such a distinction is difficult to see without overlaying P with another low-*Z* element (fig. S2).

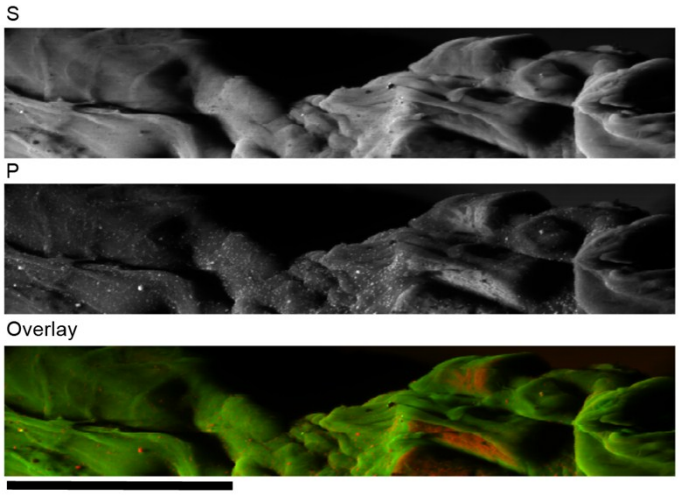


Figure S3: Elemental maps of P and S in *M. musculus* distal radius and ulna and carpal bones. Scan area is the same as in figure 1. S is evenly distributed throughout the soft tissues, while P is concentrated mostly in the ossified tissues. This correlation can only be viewed in the overlay of S (green) and P (red). Scale bar is 1 mm.

3. Additional Synchrotron XRF High-Z Maps

These maps represent elements detected in the full EDS spectra, but whose distributions could not be visually correlated with biological structures.

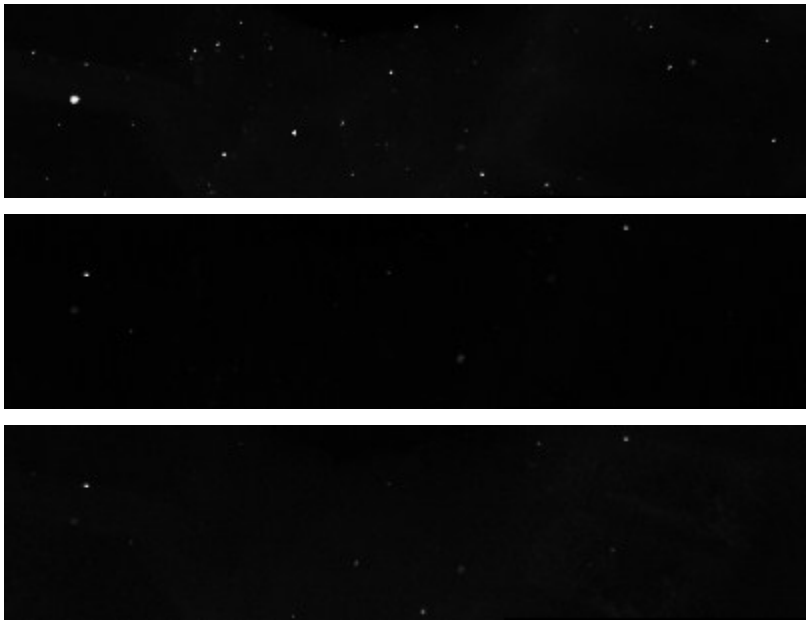


Figure S4: From top to bottom, elemental maps of Cu, Fe and Ni in *M. musculus* distal radius and ulna and carpal bones. Scan area is the same as in figure 1.

4. Additional Standards for Synchrotron-Based XRF

The Durango apatite standard used to calibrate and quality check the quantification data fits for the *M. musculus* specimen has been analysed using a multitude of techniques including synchrotron XRF, microprobe (which uses Durango apatite as a standard) and ESEM. Below we compare the concentrations of elements in Durango apatite presented in this study against other geological standards JA-3 and JG-1A also analysed at the Diamond Light Source synchrotron (DLS), beamline I-18. Results show that Durango apatite is well constrained and a reliable standard to use in synchrotron XRF analyses (table S1) and that the concentrations presented in the study are reliable.

Durango Apatite Standardization

	2016 session SR-XRF	2015 session SR-XRF	2014 session SR-XRF	EPMA	Bead XRF	PIXE XRF	Durango Apatite[xx]	Durango Apatite[yy]
P	18.20 %	n.a.	18.74 %	18.20 %	17.50 %	n.a.	17.8 %	18.1
Ca	38.20 %	38.19 %	38.80 %	38.20 %	38.80 %	46 %	38.61 %	38.72
Fe	540	397	621	280	203	333	420	430-510
Zn	40	27	49	b.l.d.	80	35		47
Sr	1400	669			516	982	510	70-915
La			5078	4154				2895-4210
Ce			7802	5869			4440	270-5640
As			1186		1100*			95-1050

*Laser ablation ICP-MS

[xx] Jarosewich et al.[1]

[yy] Marks et al.[2]

Glass Bead XRF Standardization (all oxide wt. %)

	JA3		JG1A	
	Bead XRF	Reference	Bead XRF	Reference
SiO_2	62.74	62.26	73.79	72.19
TiO_2	0.64	0.68	0.18	0.25
Al_2O_3	15.55	15.57	13.77	14.22
Fe_2O_3	6.50	6.59	1.95	2.05
MnO	0.11	0.11	0.07	0.06
MgO	3.49	3.65	0.58	0.69
CaO	6.31	6.28	2.14	2.13
K_2O	1.40	1.41	4.08	4.01
Na_2O	3.16	3.17	3.36	3.41
P_2O_5	0.11	0.11	0.08	0.08

Table S1: Synchrotron quantification of trace elements for the Durango apatite used in this study (top) given in ppm or weight percent (%). Techniques include Synchrotron- Radiation X-Ray Fluorescence (SR-XRF), Electron Probe Micro Analysis (EPMA), Bead XRF, Proton Induced X-ray Emission (PIXE) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Quality checks were accomplished using the glass bead method by analysing two geological standards, JA-3 and JG-1A (bottom), in addition to the Durango apatite. Error for synchrotron quantification is ~10% of the absolute value.

5. Quantification for *M. musculus*

Synchrotron

To quantify element concentrations, multiple point analyses were undertaken by locating an area of interest within the scan, driving the stage to the area, and collecting a full energy spectrum for 30 sec. Multiple point analyses are taken per area of interest to account for variation within the sample. The setup at the Diamond Light Source allows for the exact motor positions to be saved for each pixel, allowing for precise locations to be selected using the viewed elemental maps. Point analyses are then processed through the PyMCA software[3] ~~[1]~~, which is used to fit point spectra based on the raw EDS files and from the experimental parameters using a Durango apatite standard of known elemental concentrations for calibration.

A 25 µm attenuation layer of keratin was added to account for the attenuation caused by soft tissue still on the sample. This is based on the results found in the Environmental Scanning Electron Microscope (ESEM) when looking at the attenuation of Ca and P. The influence of the organic content within the bone and cartilage had to also be considered in order to calculate elemental concentrations. Although elements associated with organics (H, O, C, N) are too light to be detected by the set up at DLS, they do influence the stoichiometry of the sample matrix, which is used by the PyMCA software in determining trace element concentrations.

ESEM

Based on the attenuation of the Ca and P fluorescence from the bones, this layer must be 25 microns thick and has a stoichiometry similar to keratin. These variables were added as a filter layer when analysing the synchrotron XRF data in PyMCA.

Element	Durango Standard	*M. musculus* soft tissue
C		53.9%
N		13.59%
O		28.36%
Si		1%
P	16.69%	1.34%
S		0.55%
Cl		0.58%
K		0.64%

Table ~~S1~~S2: ESEM XRF quantification of trace elements in one-day postnatal *M. musculus* forelimb soft tissue given in weight percent (%). Calibration was accomplished using a Durango apatite standard.

References

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