PERSPECTIVES ON CONCORDANCE BETWEEN MICRO-CT AND TRADITIONAL STAINING METHODS.

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Link

A NOTE ON THE STAINING OF THE SKELETON OF CLEARED SPECIMENS WITH ALIZARIN RED S

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ABSTRACT.—**A selective, progressive method for staining the skeleton in cleared specimens, developed with rat material.**

Fix in 95% alcohol for at least 48 to 96 hrs. Even longer fixation is desirable. Then place in a 1% solution of KOH until the bones are clearly visible through the surrounding tissues. Transfer directly to a dilute solution of alizarin in KOH, one part alizarin to 10,000 parts of 1% KOH. Allow the stain to act until the desired intensity is attained. Fresh stain may be added if necessary.

Complete the clearing process, (1) in Mall's solution, water 79 parts, glycerine 20 parts and KOH 1 part; (2) in increased concentrations of glycerine. Store in pure glycerine.

The success of the method depends on obtaining the proper degree of clearing before staining. If the specimen is insufficiently cleared, a general staining of all tissues usually occurs.

“Staining” stage of skeletal staining process – may/may not be preceded by maceration step.

Rat

Lab 1	1 % KOH, 0.003% alizarin red (auto, 30^{o}C)
Lab 2	1.5% KOH, 85% IDA, 0.002% alizarin red (manual and auto, 30^{o}C)
Lab 3	1% KOH, 0.005% alizarin red (auto, 30^{o}C)
Lab 4	[No KOH] 0.003% alizarin red (manual)
Lab 5	1% KOH, 0.05% alizarin red (manual)
Lab 6	1% KOH, 0.004% alizarin red (manual)

Alizarin red S stains the calcium present in ossified bone but an excess can leave background staining that requires clearing, hence, variations have developed as each laboratory attempts to “tweak” the original method and improve on it. Differences in the clearing process also exist with a variety of clearing agents being used. Specimens may be processed automatically at a pre-determined temperature or manually at ambient temperature.

Rabbit

Lab 1	2% KOH, 0.007% alizarin red (auto, 30^{o}C)
Lab 2	1.5% KOH, 85% IDA, 0.003% alizarin red (manual and auto, 30^{o}C)
Lab 3	1% KOH, 0.005% alizarin red (auto, 30^{o}C)
Lab 4	[No KOH] 0.003% alizarin red (manual)
Lab 5	[No KOH] 0.05% alizarin red (manual)
Lab 6	1% KOH, 0.004% alizarin red (manual)

Differences in alizarin red S staining intensity and varying clarity of the specimens from different laboratories noted

KOH = potassium hydroxide, IDA = Industrial Denatured Alcohol

FACTORS THAT CAN INFLUENCE STAINING

- Preservative (or fixative!) used for specimens prior to processing (formalin, various % alcohol concentrations, thymol water)
- How quickly processing is started after PM
- Temperature at which processing is conducted
- How quickly after processing examination begins
- Other factors - different strains, different day of gestation for necropsy, etc.

FACTORS THAT CAN INFLUENCE OUTCOME

- Training/experience
- Use (or not) and degree of magnification
- Subjective judgement
- What is ‘normal’? Assessment is made against a definition of ‘normal’ that is essentially arbitrary and is not provided in any standard reference text.
- Calling levels against ‘normal’ - defined within their own laboratory

EXAMPLES

Cervical neural/vertebral arches

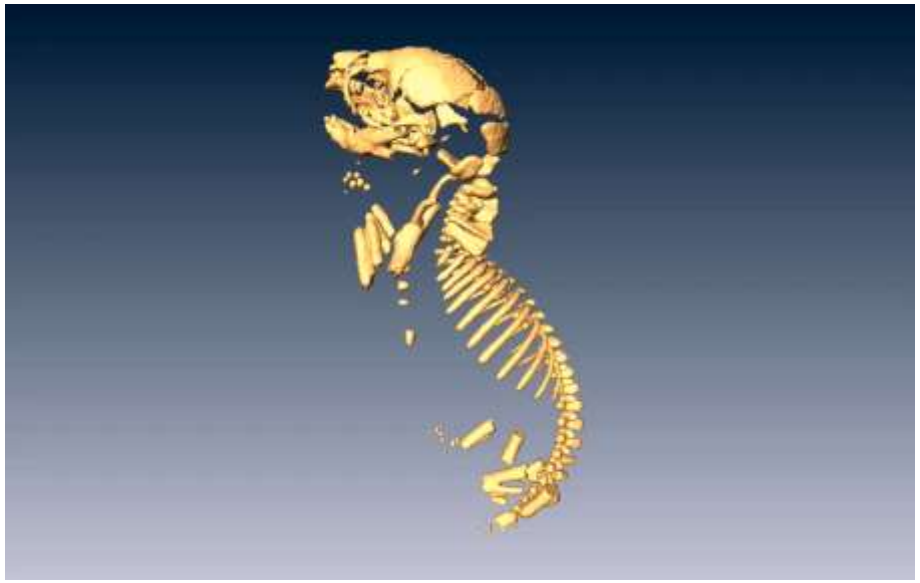
- by individual arch
- ‘n’ arches may have to be affected before an observation is recorded
- ‘n’ may depend on which arches are affected e.g. 1 and 2, 3 – 7

Supernumerary (thoraco-lumbar) ribs

- appearance, or length or both
- different terms used to describe each “class” (vestigial, short, rudimentary, etc.)

Incomplete ossification

Incomplete ossification may be used to describe the appearance of a bone in one laboratory where as in another the same appearance would be classed as within normal limits and an observation made only when no ossification (staining) was evident.



The point of all of this is to demonstrate that skeletal examination results data should not be regarded as a one homogenous standard against which all micro-CT results can be compared for concordance purposes.

Work to show concordance between results obtained following alizarin red staining and micro-CT imaging within an individual laboratory could (and has) been carried out. However, for the reasons already stated concordance “standards” are likely to differ between individual laboratories.

OPPORTUNITY!!

Consider some sort of standard imaging regime (“phantoms”) for different species on different days of gestation (equivalent to everyone following the same alizarin red staining method, under the same conditions)?

Fetal morphologists from different laboratories work together to define “normal” for each of these conditions, collaborate to define “calling levels” and assign appropriate observations, using agreed terminology (Makris)? To facilitate and maintain consistency image sets could easily be shared between laboratories who would then examine them and compare the results.

Advantages would be homogeneity of the resulting data, allowing a bigger pool of background data to be made available to assist in interpretation of the results and comparison of the skeletal examination results from different test articles of the same chemical class from different labs would be easier.

One more thought…..

If fetal morphologists were asked to look at the skeleton of a species which they were not familiar with the likely process would be (once a staining regime had been established) to define “normality” for the species/strain taken on a particular day of gestation.

Once the normal range for the appearance of each bone has been established it is then possible to start describing and recording observations that differ from the appearance that would be expected i.e. define calling levels.

Without anything to compare the “new” skeleton with there would be no concordance exercise yet this would still be acceptable.

So in many way the process that could follow with micro-CT already exists.

CONCLUSIONS

- Staining the fetal skeleton with alizarin red S and clearing the remaining soft tissue is the (almost?) universally used and accepted (Gold Standard) method for specimen preparation.
- Inconsistency in specimen preparation and examination ("calling levels") has arisen between different labs.
- Skeletal examination results data should not be regarded as a one homogenous standard.
- The introduction of micro-CT and standardised terminology, together with closer collaboration between users, may offer an opportunity to produce a tighter dataset, without inconsistency.
- Finally – prepare to be lead by the fetal morphologists, after all they are the people that know the specimens best!