INTRODUCTION: Skeletal morphology in mice is typically evaluated using alizarin red S- and alcian blue-stained preparations (skeletal preparations). This assay provides a three-dimensional (3-D) visualization of the bone (alizarin red S-stain) and cartilage (alcian blue-stain) that can be viewed under a microscope. Although the 3-D structures are preserved using this type of preparation, only two-dimensional (2-D) color images of the skeletal structures can be obtained using a microscope, which makes 3-D quantitative morphometry impossible. Skeletal preparations are time consuming, labor intensive, and prone to damage during processing and handling. Finally, the preparation is a destructive one, such that the specimen cannot be used for other experiments. The goal of this study was to use high-resolution micro-CT imaging and 3-D reconstruction to chart the chronology of mouse skeletal development and to explore the capability of this method to provide information about specific regions of the skeleton that may be difficult to identify by other means.

METHODS: All animal procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committee. Mice of the C57Bl67 strain (Charles River laboratories, Wilmington MA) were maintained and bred under defined light-dark conditions with unlimited access to standard lab chow and water. The appearance of a vaginal plug on the morning after mating was taken as day 0.5 of gestation. Plugged mice were killed by overdose of carbon dioxide at defined periods of time after conception and the embryos were fixed in 70% ethanol for 24-48 hours. The embryos were embedded in paraffin using routine automated processing methods. Paraffin-embedded embryos (E12.5-E18.5, at 1 day intervals) were mounted on bone wax in the x-ray beam for micro-CT imaging. High-resolution images (35 μm) of the samples were obtained by collecting one-hundred and eighty 5122 12-bit projection radiographs at 1° intervals around half of the entire specimen. These images were collected at approximately 24 kVp, 1000 μA, and 1 sec exposure time with the image intensifier operating in 7-inch mode. The micro-CT images were pre-processed for intensity and spatial non-uniformities. The 3-D reconstructed volumes (5123) were obtained using a tent-FDK filtered back-projection method based on a circular data acquisition [1].

For alizarin red S- alcian blue-stained skeletal preparation, we followed the method of C. Amott as described by Kaufman [2]. To determine initially if the micro-CT imaging was of comparable sensitivity to alizarin red S- alcian blue-staining, we imaged an embryo by micro-CT (Figure 1A) and subsequently made a skeletal preparation (Figure 1B). The stained-skeletal preparation was then re-imaged by micro-CT (Figure 1C).

RESULTS: In Figure 1, an 18.5 day-old mouse embryo is used to demonstrate that micro-CT imaging can be used to successfully identify all mineralized structures stained with alizarin red S in the skeletal preparations. The mineral is highlighted in red in these images and can be used for qualitative and quantitative comparison. Furthermore the skeletal preparation could then be successfully re-imaged without significant loss of resolution. In fact, the 3-D reconstruction of the skeletal preparation, showed considerable additional detail not obtainable from the preparation using optical means alone, such as the surface texture of the bones. Since many such surface landmarks serve as muscle and tendon attachment points, micro-CT could help to identify defects of tendon and bone integration. The implications from this figure are that micro-CT is a satisfactory means of resolving the bony skeleton and that even archival skeletal preparations can be reanalyzed if required to obtain the benefit of 3-D reconstruction and texture resolution that micro-CT makes possible. However, unlike skeletal preparations where cartilage is well-identified by the alcian blue stain, cartilage does not have sufficient radio-opacity to allow its visualization by micro-CT.

DISCUSSION: Our studies demonstrate that 3-D micro-CT imaging of embryos is a sensitive, rapid and facile tool for analysis of mouse development. In addition to the obvious uses for identifying defects of skeletal patterning or bone mineral density, we feel that there are a number of other exciting applications. Morphometrics, such as in skull and spinal canal, will disclose neural compression of bony origin, and analysis of the interrelationship between soft-tissue and skeletal growth. The high resolution imaging of surface textures such as is shown for the interior of the cranial cavity and craniofacial structures approaches that which might be provided by low magnification scanning electron microscopy, but without the need for special cleaning or preparation of samples. Finally, the possibility of virtually “isolating” individual skeletal elements, such as the auditory ossicles, will be invaluable in investigating gene effects on hard-to-image sites.

REFERENCES:

ACKNOWLEDGEMENTS: This work was partially supported by a grant NIH AR47074.