Clearing and photography of whole mount X-gal stained mouse embryos

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Advances in genetic manipulation in mice have made knockout, knock-in, and transgenic mice highly useful for elucidation of gene function and structure. In many cases the mice are engineered to include a reporter gene, for example, β-galactosidase (*lacZ*) or green fluorescent protein (GFP). The reporter gene enables visualization of the expressing cells and tissues in both the heterozygous and mutant animals. When looking for promoters and enhancers that direct gene expression in a spatiotemporal-restricted manner, the ability to easily detect expression derived by various genomic fragments is essential. Traditionally, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining of mouse embryos younger then 16 days (E16) is performed as a whole mount. X-gal staining of developing organs of young embryos (e.g., less than E12) is easily viewed under a dissecting microscope as the thickness of the embryo does not obscure the staining.

In older embryos (e.g., E16) stained cells close to the surface are easily detected, while deeper staining appears blurred, making sectioning and microscopic examination necessary. In addition to sectioning, organs of older embryos can be dissected, X-gal stained, and visualized on a dissecting scope (1). Here we describe two simple manipulations that increase the visibility of the stained tissues.

During our attempts to improve photography of later-stage, alizarin red- and alcian blue-stained skeletons, we noticed that the quality of the images, the distinction between the colors, and the ability to observe the fine details are much improved when using dark field, in comparison to the conventional bright field illumination (Figure 1, A–D). Differential staining of bone and cartilage was performed according to a published protocol (2). In brief, E16.5 embryos were deskinned and eviscerated, stored in 100% ethanol, and transferred to acetone. After 48 h, they were stained overnight at 37°C in a staining solution consisting of 1 volume of alizarin red S (Sigma, St. Louis, MO, USA; 0.1% w/v in 95% ethanol), 1 volume of Alcian blue 8GX (Sigma; 0.3% w/v in 70% ethanol), 1 volume of concentrated acetic acid, and 17 volumes of 70% ethanol. The specimens were briefly rinsed in water and cleared in 1% w/v KOH, followed by 20% glycerol solution (1 volume glycerol and 4 volumes of 1% KOH) and by graded steps of glycerol/1% KOH solutions with increasing amounts of glycerol (50%, 80%, and 100%) by incubating for a week in each solution at room temperature. As expected, an E16.5 embryo became translucent, and the developing bone (red) and cartilage (blue) components were visualized (Figure 1, A and C). Pictures were taken using an Olympus SZ4045TR stereo microscope (Olympus, Hamburg, Germany) fitted with a SZH-IILD illumination base and a Nikon Coolpix 5000 digital camera (Nikon, Tokyo, Japan).

To characterize in detail the dynamic expression pattern of the transcription factor *Math1*, we took advantage of knock-in mice, in which *lacZ* has replaced the entire coding region of *Math1*, so that *lacZ* expression is controlled by the intact *Math1* locus (3). *Math1* heterozygous embryos at E16.5 were stained as a whole mount by X-gal and photographed using top illumination. As seen in Figure 1, E–F, an E16.5 embryo is not translucent, and therefore the most visible stained regions are close to the surface. These include for example the joints and Merkel cells in the base of the whiskers and dotted touch domes in the skin (3). However, staining in the joints, for example, appears hazy, and the exact location of the stained cells can be revealed only by sectioning. We sought to adopt the clearing procedure used to visualize skeletal staining and utilize it after X-gal staining, taking precautions not to dissolve the blue color precipitates.

To clear X-gal stained embryos, the following protocol was developed: whole E16.5 embryos were stained by X-gal, postfixed overnight at 4°C in 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS), and stored refrigerated in 70% ethanol (3). The embryos were cleared directly (without acetone and KOH treatments) in a series of solutions containing increased glycerol and decreased KOH concentrations. The clearing solutions contained 20%, 50%, 80%, and 100% glycerol (v/v) brought to the final volume by 1% KOH (w/v). Each embryo was placed in a 20-mL glass scintillation vial and was incubated in 15 mL clearing solution at 30°C with gentle shaking. Each incubation step lasted 4–7 days, after which the solution was changed. To avoid reflection while photographing, each embryo was transferred to a Petri dish containing enough 100% glycerol to completely cover it. Bottom bright field illumination indeed indicated that the embryos had become translucent, without compromising the quality of the X-gal staining (Figure 1, G and I). Reporter gene expression in internal tissues like the spinal cord was clearly visible. Moreover, the patchy staining in the joints was localized accurately in respect to the calcified bone that appeared darker. Having previously observed that dark field illumination improved the visualization of the skeletal staining, we similarly examined the X-gal stained embryos under dark field (Figure 1, H, J, and L). While the X-gal staining product still appeared blue, the bones shined in white, and the relative localization of the stained regions and the bones improved.

We next X-gal stained *Math1*$^{lacZ/+}$ embryos at various developmental stages (intact E12.5 and E14.5 embryos and deskinned E16.5 and E18.5 embryos) in order to indicate what ages would benefit from applying the clearing protocol (Figure 2). Additionally, we have tested various incubation periods and found that each step could be shortened to three (but not less) days, allowing the entire protocol to be completed within 12 days. At all ages tested, the embryos became clear while maintaining the X-gal staining. Clearing allowed sharper observation of stained tissues (e.g., joints and intestine at E16.5 and E18.5). When a cellular resolution of stained tissue is of interest, embryos are sectioned and microscopically examined, and therefore clearing is not essential. However, to verify that treating the embryos in KOH and glycerol does not compromise the histology and lead to leakage of the color precipitates, we compared sections from cleared and noncleared embryos. Embryos were washed in PBS, sunk in 30% w/v sucrose, embedded in OCT (Tissue Tek), and frozen (1). Sections (16–20 µm) were counterstained with eosin Y (Sigma) (1) and photographed under an Axioskop2 microscope (Carl Zeiss, Jena, Germany). Similar staining patterns were observed in various cell types, such as dorsal neurons in the spinal cord (E12.5 and E14.5) and Merkel cells around the whiskers (E16.5 and E18.5) at low (E14.5 and E16.5) and high (E12.5 and E18.5) magnification. It should be noted, that when interested in localizing X-gal stained tissues in respect to the skeleton (e.g., in the joints or vertebrae) dark field illumination may be of help, as it improved the contrast of the skeleton in embryos older than E14.5 (not shown). We therefore concluded that the shorter clearing protocol could be applied to various embryonic stages without compromising the X-gal staining or the histology.

We are aware of a different clearing protocol using benzyl alcohol and benzyl benzoate (BA/BB) (1). However, when clearing with BA/BB, X-gal staining is lost within a day,

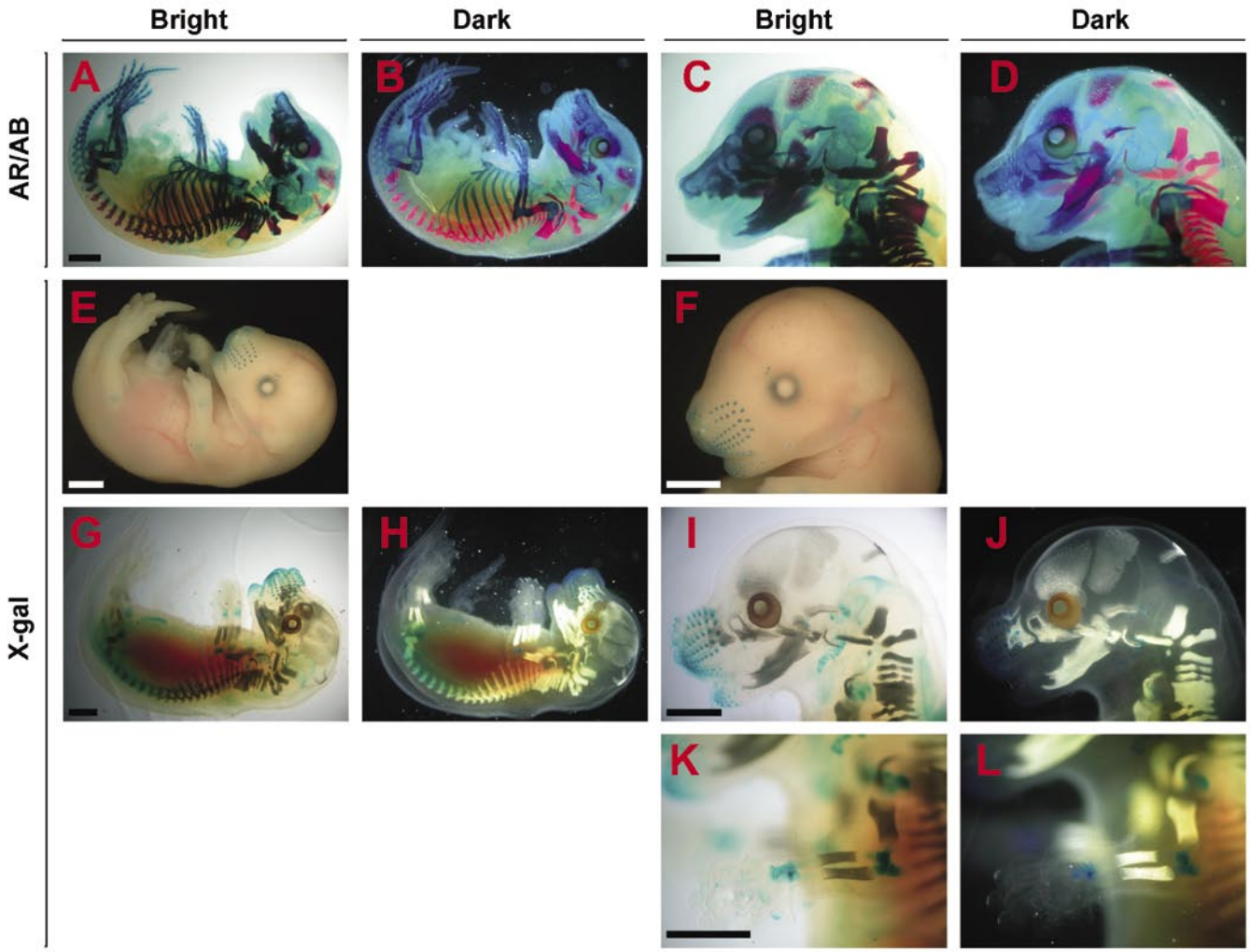

Figure 1. Visualization of skeletal and X-gal staining. E16.5 wild-type embryos were simultaneously stained by alizarin red and alcian blue to visualize the bone and cartilage, respectively (A–D). *Math1*$^{lacZ/+}$ E16.5 embryos were stained by X-gal (E–L) and photographed in 70% ethanol (E and F) or in 100% glycerol after clearing (G–L). Bright field illumination was applied below (A, C, G, I, and K) or above (E and F) the specimen. Dark field illumination was applied when indicated (B, D, H, J, and L). Scale bar, 2 mm. X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

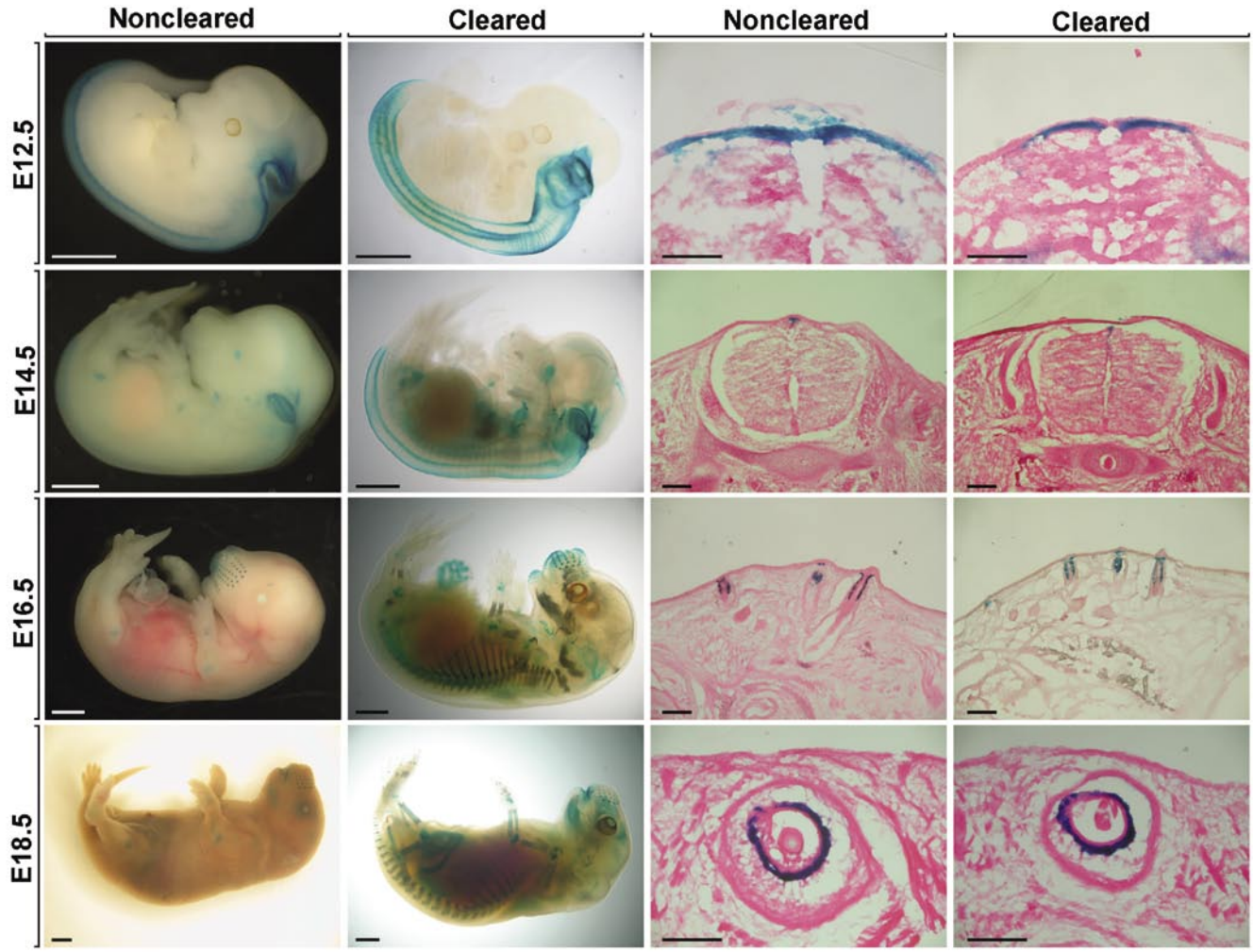

Figure 2. Clearing of embryos at various stages does not compromise histological details. *Math1*$^{lacZ/+}$ embryos at E12.5, E14.5, E16.5, and E18.5 were cleared as described and photographed under bright field illumination. Cryoprotected embryos were sectioned and counterstained with eosinY. Shown are sections through the spinal cord at the forelimb level (E12.5 and E14), lower lip (E16.5), and snout (E18.5) displaying similar histology in noncleared and cleared embryos. Scale bars, 2 mm (whole embryos) or 200 µm (sections).

making it impossible to take more pictures at a later stage, therefore store the specimens or send them for an expert evaluation. Thus, although taking longer to achieve, but with minimal hands-on requirements, simple glycerol/KOH clearing of embryos and dark field illumination are of benefit when examining and photographing transgenic, knockout or knock-in embryos engineered to contain a *lacZ* reporter.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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