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Quantification of Methylene Blue Exclusion for Tracking of Regenerative Re-Epithelialization

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A major difference between the non-regenerative (scar-forming) wound closure found in mammals and the regenerative wound closure (scarless healing) found in many fish and salamanders is that re-epithelialization of the injury is much faster in animals that can regenerate appendages [1]. This re-epithelialization, in turn, is essential for appendage regeneration. Methylene blue dye has long been used to guide surgical debridement of burn injuries [2] because it is absorbed by damaged tissue but not undamaged or regenerated epithelium. Here, we use microscopy and image analysis to develop a quantitative methylene blue exclusion assay to assess the time course for tr-epithelialization in the regenerating zebrafish fin and axolotl tail. We expand and adapt methods used for the assessment of re-epithelialization associated with wound healing in zebrafish [3] to the epithelialization associated with tail regeneration both in zebrafish and axolotls, classic model systems for appendage regeneration.

Adult zebrafish (*Danio rerio*, n=3) and larval and juvenile axolotl salamanders (*Ambystoma mexicanum*, albino strain, n=12) were anesthetized with 0.1% benzocaine prior to tail tip amputation. The amputation plane was briefly (~3 min.) stained with 1% (w/v) methylene blue, then washed extensively in system water. Animals were digitally imaged under the same settings using a Bausch & Lomb dissecting microscope until methylene blue was no longer visible. Since methylene blue is absorbed only by damaged tissue [2], its exclusion was used to assess re-epithelialization. The amputation plane was selected as the region of interest and thresholded for methylene blue labeling using ImageJ, version 1.49 [4]. The percent area of methylene blue within the amputation plane was plotted versus time. Animal procedures were in accordance with Towson University IACUC protocol 12052013-RD1.

As expected, there was little methylene blue staining in animals that had not undergone amputation, nor was there staining of uninjured tissue in amputated animals (Fig. 1). Methylene blue strongly labeled the tissue injured by amputation. Staining was most intense immediately post-amputation, and diminished with time as re-epithelialization progressed (Figs. 2, 3). Because it is a vital dye, methylene blue can be used to track regenerative re-epithelialization over time within individuals (e.g., Fig. 2). Measurements of tail outgrowth length and snout vent length in subsequent weeks indicated that tail regeneration rate and body growth was not altered by methylene blue treatment as compared to control, unstained animals. No morphological abnormalities were observed. Re-epithelialization of the amputated tail, as assessed by methylene blue exclusion, occurred quickly, within ~2h in all groups and the time course was highly reproducible across individuals. The process was more rapid in zebrafish than the salamanders (Fig. 3A vs 3B). The time frame for *in vivo* re-epithelialization in regenerating salamander appendages was more rapid than in the *ex vivo* salamander model of wound healing [5], suggesting that the explant wound healing model may underestimate how quickly regenerative re-epithelialization occurs *in vivo*. Regenerative re-epithelialization in zebrafish was also more rapid than in the zebrafish wound model [3]. The developed techniques allow us to compare the time course for regenerative re-epithelization across different strains and stages, as well as under the influence of various pharmacological inhibitors that affect regeneration.

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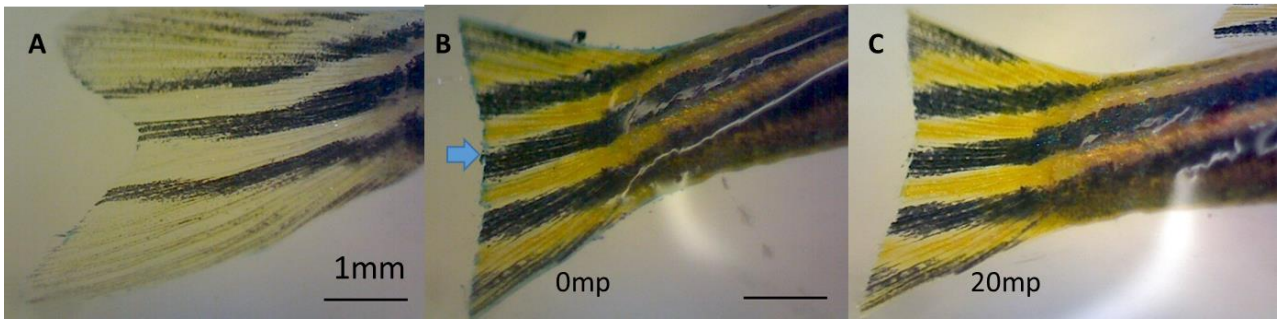

Figure 1. Methylene blue is not absorbed by undamaged zebrafish fin (A) but is absorbed by tissue damaged by amputation (B, arrow points to blue staining at amputation plane). Images recorded immediately following methylene blue staining procedure. mp=minutes post-methylene blue.

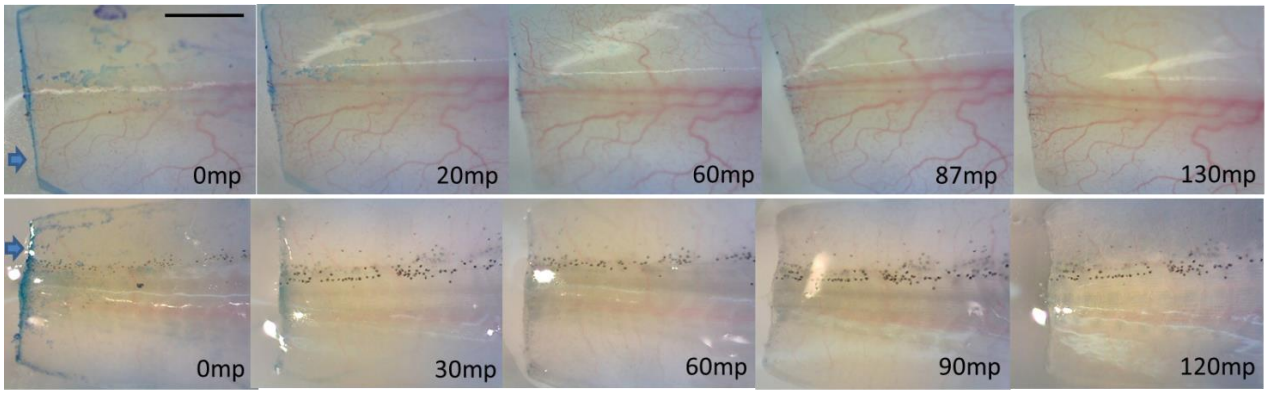

Figure 2. Methylene blue exclusion in individual larval (top row) and adult albino salamander (bottom row). Arrow points to staining at amputation plane. Bar=1mm. mp=minutes post-methylene blue.

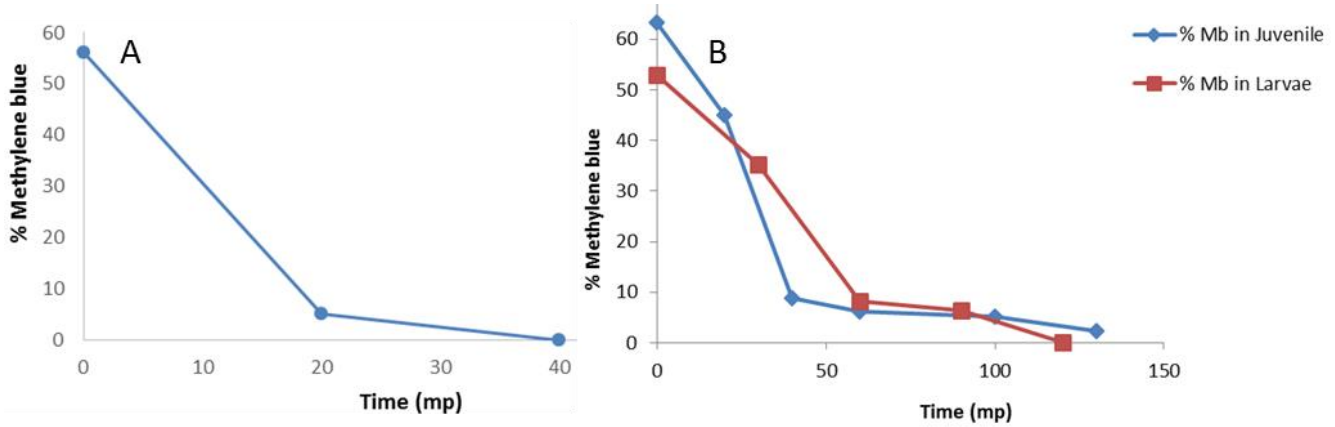

Figure 3. Percent area of methylene blue within the amputation plane diminishes with time in adult zebrafish (A) and juvenile and larval salamanders (B) as the tail re-epithelializes.

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