CONTRACTION WAVES CAUSED BY ELECTROSTIMULATION OF XENOPUS LAEVIS EMBRYOS

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INTRODUCTION
During human embryogenesis, genetic or mechanical departures from typical patterning events can result in birth defects. One specific complication is spina bifida, the incorrect closure of the backbone and its associated membranes. Humans living with spina bifida often need to coordinate with a multidisciplinary team of specialists during their lifetime in order to manage the psychological, social, and physical difficulties associated with living with the disease. Additionally, the combined medical costs between all spina bifida patients in the US are upwards of $200 million per year [1]. Even while spina bifida affects up to nearly 1500 newborns each year alone [2], it is still only one of the many malformations that can occur during human development. Understanding the mechanisms of development could better enable the scientific community to mitigate the prevalence of birth defects in the future.

Frog embryos from the species Xenopus laevis serve as a particularly advantageous animal model for studying embryogenesis, due to their observable development outside of the mother and their status as a vertebrate. The mechanical forces that guide morphogenesis, or the movement of cells into specific spatial patterns, can be examined in Xenopus to better understand how development mechanistically occurs. Contraction, or the force of cell shrinking, has been connected to both neurulation and wound healing events [3,4,5].

Therefore, examining the molecular mechanisms that drive contraction in Xenopus laevis embryos would serve to illuminate potential processes that drive particular patterning events. Such examinations have already begun: transient calcium waves have been identified in Xenopus laevis embryos during convergent extension [6]. Additionally, Eric Weston, a prior undergraduate at the University of Pittsburgh, showed that electrostimulating the mesenchymal layer of Xenopus explants resulted in the initiation and propagation of contraction waves. However, this tissue was not stimulated on the same surface from which it was imaged.

OBJECTIVE
The first objective of this research project was to build an electrostimulation apparatus that could stimulate Xenopus laevis animal cap explants from the epithelial surface, rather than from beneath the mesenchymal layer. The second objective was to determine calcium’s role in the propagation of the contraction “waves” in response to the electrostimulation.

HYPOTHESIS/SUCCESS CRITERIA
The primary success criteria of this research project were as follows: (1) Quantification of time-lapse imagery data, (2) repeatable stimulation of contraction with electricity, (3) survival of >50% of embryos after calcium chelation up through stage 10, and (4) a sufficient sample size throughout experimentation. My hypothesis was that chelating calcium in Xenopus embryos would result in a significant diminishment of contraction wave area in response to electrostimulation.

METHOD
Embryo preparation: Animal caps were explanted from Xenopus laevis embryos at stage 10.5 after 24 hours of 14°C incubation.

Electrostimulation: An Ag/AgCl [1] wire carried a pulse of charge onto the explant and into a platinum/iridium grounding wire placed at an arbitrary distance (>1cm). The Ag/AgCl electrode was placed inside of and positioned with a DFA-filled glass pipette within a micromanipulator (Fine Science Tools Inc., M3301R). A 1.0mm OD x 0.78mm ID x 100mm L glass capillary (Harvard Apparatus, GC100T) was pulled using a pipette puller (Sutter Instrument Co., P-97) at various recipes to procure a pipette with optimal tip diameter (60-70 um). Embryos were stimulated with a 4ms, positive monophasic pulse using a stimulator (Grass Astro-Med Inc., S88X) and a photoelectric stimulus isolation unit for a constant current output (Grass Technologies, PSIU6). The explant responses were captured using a camera adapted to a microscope (Zeiss Stemi, 2000-C) and analyzed with ImageJ.

Calcium chelation: BAPTA (tetrpotassium salt, cell impermeant, Molecular Probes, B-1204) was dissolved in distilled water and was injected using glass pipette needles at the 1-, 2-, or 4-cell stage with rhodamine dextran 10K (RDA10K) as a fluorescent tag to verify injection. The RDA10K was combined with a 0.1125M concentration of BAPTA in a 1:1 (v:v) ratio prior to injection to achieve a fluorescent BAPTA concentration of 0.05625M. The volume of the embryo was assumed to be 0.9uL in calculating BAPTA concentrations in vivo. Injection volumes ranged from 4-16nL.

Parameters: Contraction area was calculated by using equation 1:

\[
\%\text{Contraction Area} = \frac{\text{contraction area}}{\text{explant area}} \times 100
\] (1)

The contraction area value was calculated by hand-drawing a polygon around the maximal contraction area in ImageJ – this analysis was blinded as to prevent bias from impacting the size of the area measurements. Survival rate of BAPTA injections was calculated as the percentage of embryos that did not lyse or fail to divide up through stage 10.

Statistics: Groups were compared using a two-tailed two-sample t-test, and a p-value of <0.05 indicated significance.
RESULTS
Stimulating the embryos with a variety of currents yielded contraction areas (%), as can be seen in Figure 1.

![Graph showing contraction areas of Xenopus laevis after different stimulation methods.](image)

Figure 1. Contraction areas of *Xenopus laevis* after different stimulation methods. Each bar represents a different applied current. No statistical significance was achieved between methods (p>0.05).

While statistical significance could not be achieved due to sample size, the method producing the largest contraction area (15mA) was selected for further animal cap stimulations—a larger area would allow for any diminishment in contraction area to be seen.

The survival rate of BAPTA-injected embryos tended to decrease as the concentration of the chelator in the embryo increased. There was 83% survival for the uninjected group, 50% survival for the 0.25mM group, 53% for the 0.50mM, and 36% for the 1.00mM. All samples injected with BAPTA eventually exogastrulated. Because 0.50mM was the highest concentration that resulted in a greater than 50% survival rate up through stage 10, 0.50mM BAPTA was chosen as the selected treatment for injections to follow.

The results for the contraction responses to stimulation after chelation with BAPTA are summarized in Table 1. Due to time constraints, two trials were conducted per group. The calcium-chelated explants tended to have diminished contraction areas in comparison to the uninjected control group. Significance could not be achieved (p>0.05).

<table>
<thead>
<tr>
<th>Trial</th>
<th>Uninjected</th>
<th>0.50mM BAPTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>66</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>11</td>
</tr>
</tbody>
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Table 1. % Contraction Areas for Uninjected and BAPTA-Injected Xenopus explants

DISCUSSION
There was no statistically significant difference between contraction areas of explants that were stimulated with different currents. While larger currents did tend to induce larger contractions, this correlation cannot be confirmed unless larger sample sizes are used in the future.

The decrease in survival rate as the concentration of BAPTA increased suggests that BAPTA successfully lowered the levels of available ions that are required for successful embryogenesis. Because calcium transients are understood to be present during development [6], and BAPTA is a chelator for calcium, it follows that calcium suppression was likely occurring in the embryos. Because the fluorescence could only verify BAPTA injection rather than BAPTA activity levels, though, the extent of calcium suppression could not be confirmed. In future studies, a control test should be performed to verify whether or not available calcium levels within the embryo have decreased in response to binding with BAPTA.

Regarding the contraction response of explants from BAPTA-injected embryos vs. uninjected embryos, the data collected thus far matches the hypothesis that calcium chelation drives contraction wave diminishment. Chelated explants had smaller contraction waves (11-19%) than uninjected embryos (20-66%), although the insufficient trial size of 2 limits the veracity of such a conclusion.

With respect to the initial goals of the experimentation, three of the four success criteria were met.

1. The images obtained during testing were able to be quantified. (2) The electrostimulation did produce a contractile response each time electricity was delivered. (3) Finally, at least 50% of the embryos treated with 0.50mM BAPTA survived the treatment up through stage 10. A sufficient sample size to support significance, however, was not met throughout experimentation. Consequently, the hypothesis that calcium chelation with BAPTA results in significant diminishment of contraction area after electrostimulation cannot be properly assessed until a larger sample size is used. The connection between developmental calcium transients and contraction waves cannot yet be confirmed by these results.

Future directions include increasing the sample size of stimulations, as well as applying this electrostimulus technology towards whole embryo investigations without the need for microsurgery or explants.

ACKNOWLEDGMENTS
I’d like to thank Dr. Lance Davidson for his mentorship, as well as Dr. Holley Lynch, Deepthi Vijayraghavan, Tim Jackson, Joe Shawky, and Dr. Carsten Stuckenholz for their training and guidance pertaining to embryo care and treatments. I’d also like to thank Dr. Tracy Cui, Xin Zheng, and Zhanhong Du for guidance concerning electrostimulations.

REFERENCES