

Submission ID #: 70345

Scriptwriter Name: Sulakshana Karkala

Project Page Link: <https://review.jove.com/account/file-uploader?src=21455273>

**Title: Morphometric Analyses of Shape: The Analysis Software
Toolbox for Craniofacial Shape Quantification in Zebrafish**

Authors and Affiliations:

Makenzie C. Stearsman¹, Jennan A. Lahamer¹, Raèden Gray^{1,2}, C. Ben Lovely¹

**¹Department of Biochemistry and Molecular Genetics, Alcohol Research Center,
University of Louisville**

**²Department of Pediatrics, Section of Developmental Biology, University of
Colorado School of Medicine, Anschutz Medical Campus**

Corresponding Authors:

C. Ben Lovely (ben.lovely@louisville.edu)

Email Addresses for All Authors:

Makenzie C. Stearsman (makenzie.stearsman@louisville.edu)

Jennan A. Lahamer (jennan.lahamer@louisville.edu)

Raèden Gray (raeden.gray@cuanschutz.edu)

C. Ben Lovely (ben.lovely@louisville.edu)

Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

- 3. Filming location:** Will the filming need to take place in multiple locations? **No**

- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

Current Protocol Length

Number of Steps: 27

Number of Shots: 56

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Makenzie Stearsman:** Our research uses morphometric software to quantify whether ethanol and *bmp7a* interact to cause subtle craniofacial defects in zebrafish.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Makenzie Stearsman:** Linear measures miss subtle changes. With this method, we use landmarks to quantify shape changes while controlling for size differences.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.3. **Makenzie Stearsman:** Consistent sample mounting is the biggest challenge; tilted larvae can cause inaccurate measurements and alter results.
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Makenzie Stearsman:** This protocol adapts to different anatomical structures, analyzes 3D data, generates linear measures, and helps determine experimental sample sizes.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Makenzie Stearsman:** Future studies will investigate other genotype ethanol sensitivities, expand sample sizes, and apply this versatile toolbox to various anatomical structures.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

What problem in your field of research does your article address?

- 1.6. **Makenzie C. Stearsman, PhD Candidate**: (authors will present their testimonial statements live)

1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What value do you expect this video article to have for other researchers working in your field?

- 1.7. **Makenzie C. Stearsman, PhD Candidate**: (authors will present their testimonial statements live)

1.7.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Louisville

Protocol

2. Geometric Morphometric Analysis Using tpsDigs2 Software

Demonstrator: Makenzie Stearsman

- 2.1. To begin, open the tpsDigs2 (*T-P-S-Digs-Two*) software [1]. Click **Input source**, then **File**, and select the saved tps Notepad file [2].
 - 2.1.1. WIDE: Talent seated at a computer and launching the tpsDigs2 software.
 - 2.1.2. SCREEN: 70345_screenshot_1.mp4 00:02-00:10
- 2.2. Click **Options** to visualize the **Image tools** option [1]. Set the reference length to 100 micrometers and press on **Set scale** [2].
 - 2.2.1. SCREEN: 70345_screenshot_2.mp4 00:04-00:13.
 - 2.2.2. SCREEN: 70345_screenshot_2.mp4 00:13-00:29.
- 2.3. Click **OK** to confirm the scale parameters [1]. Then exit the **Image tools** window [2].
 - 2.3.1. SCREEN: 70345_screenshot_2.mp4 00:32-00:33.
 - 2.3.2. SCREEN: 70345_screenshot_2.mp4 00:34-00:37.
- 2.4. Then click the **aim** symbol and place the first landmark at the midline joint between the Meckel's cartilages [1].
 - 2.4.1. SCREEN: 70345_screenshot_3.mp4 00:02-00:03.
- 2.5. Place the second landmarks at the bilateral joints between Meckel's and the palatoquadrate cartilages [1].
 - 2.5.1. SCREEN: 70345_screenshot_3.mp4 00:04-00:06.
- 2.6. Place the third landmark at the midline joint between the ceratohyal cartilages [1] and the fourth landmark at the bilateral joints between the palatoquadrate and ceratohyal cartilages [2]. Then place the fifth landmarks at the distal end of the hyomandibular cartilages, ensuring landmarks are placed in the same order for every image [3].
 - 2.6.1. SCREEN: 70345_screenshot_3.mp4 00:06-00:08.
 - 2.6.2. SCREEN: 70345_screenshot_3.mp4 00:09-00:12.

2.6.3. SCREEN: 70345_screenshot_3.mp4 00:12-00:16.

2.7. After placing landmarks, click **File** to open the dropdown menu, select **Save data** and then **Overwrite** to save the updated file [1]. Exit the tpsDigs2 software [2].

2.7.1. SCREEN: 70345_screenshot_4.mp4 00:03-00:11.

2.7.2. SCREEN: 70345_screenshot_4.mp4 00:11-00:13.

3. Geometric Morphometric Analysis Using MorphoJ Software

3.1. Launch the MorphoJ (*Morph-Oh-J*) software [1].

3.1.1. SCREEN: 70345_screenshot_5.mp4 00:04-00:08

3.2. Click **File**, select **Create new dataset** and assign a name to the dataset [1]. Click **tps (T-P-S)** and select the Notepad file containing the newly added data points, then create the dataset [2].

3.2.1. SCREEN: 70345_screenshot_6.mp4 00:02-00:18.

3.2.2. SCREEN: 70345_screenshot_6.mp4 00:19-00:32.

3.3. In **Project Tree**, click the dataset [1]. Select **Preliminaries**, then **New Procrustes fit**, choose **Align by principal axes**, and click **Perform Procrustes fit** [2].

3.3.1. SCREEN: 70345_screenshot_7.mp4 00:01-00:02.

3.3.2. SCREEN: 70345_screenshot_7.mp4 00:02-00:13.

3.4. Under **Preliminaries**, select **Generate covariance matrix** to obtain Procrustes coordinates [1]. Execute the function when prompted [2].

3.4.1. SCREEN: 70345_screenshot_8.mp4 00:04-00:17.

3.4.2. SCREEN: 70345_screenshot_8.mp4 00:18-00:20.

3.5. Now select **Create or edit wireframe** and link the points on the images [1]. Click **Link points** and accept or create the image then edit classifiers as needed [2].

3.5.1. SCREEN: 70345_screenshot_9.mp4 00:04-00:10-00:9-00:32.

3.5.2. SCREEN: 70345_screenshot_9.mp4 00:32-00:34

3.6. Open a spreadsheet program while keeping MorphoJ open [1]. Enter classifier information such as **Genotype**, **Treatment**, **Genotype and Treatment**, and **Experiment**, then save the file as a CSV (*C-S-V*) file [2].

3.6.1. SCREEN: 70345_screenshot_10.mp4 00:09-00:11.

3.6.2. SCREEN: 70345_screenshot_10.mp4 00:41-00:47.

3.7. In MorphoJ, click **File** and select **Import classifier variables** to import the CSV file [1]. Return to **Project Tree** and click the dataset [2].

3.7.1. SCREEN: 70345_screenshot_11.mp4 00:03-00:24.

- 3.7.2. SCREEN: 70345_screenshot_11.mp4 00:24-00:33.
- 3.8. Under **Preliminaries**, select **Edit classifiers** to confirm all images are included [1]. In **Project Tree**, select **Covmatrix**, then click **Variation** at the top of the page [2].
- 3.8.1. SCREEN: 70345_screenshot_12.mp4 00:06-00:11.
- 3.8.2. SCREEN: 70345_screenshot_12.mp4 00:24-00:27.
- 3.9. Select **Principal Component Analysis** to calculate principal component scores [1]. Click **PC scores** to display the generated graph [2].
- 3.9.1. SCREEN: 70345_screenshot_13.mp4 00:25-00:29.
- 3.9.2. SCREEN: 70345_screenshot_13.mp4 00:29-00:34.
- 3.10. Right click on the graph and select **Confidence ellipsis** to add the desired classifier [1]. Select **Color data points**, assign colors, and click **OK** to apply changes [2].
- 3.10.1. SCREEN: 70345_screenshot_14.mp4 00:02-00:11.
- 3.10.2. SCREEN: 70345_screenshot_14.mp4 00:11-00:29
- 3.11. Under **Preliminaries**, select **Set options for the shape graph** located at the bottom of the screen [1]. Choose **Wireframe graphs** and modify the colors of the target shape, starting shape, and numbers [2].
- 3.11.1. SCREEN: 70345_screenshot_15.mp4 00:03-00:09.
- 3.11.2. SCREEN: 70345_screenshot_15.mp4 00:18-00:23.
- 3.12. Select **Variation**, then choose **Procrustes ANOVA** [1]. Export the Procrustes ANOVA results to the previously created spreadsheet file [2].
- 3.12.1. SCREEN: 70345_screenshot_16.mp4 00:04-00:14.
- 3.12.2. SCREEN: 70345_screenshot_17.mp4 00:04-00:08 and 00:44-00:49.
- 3.13. In MorphoJ, select the original dataset in **Project Tree** [1]. Click **Comparison**, then **Canonical Variate Analysis**, select the classifier variables **Genotype and Treatment**, and execute the function [2].
- 3.13.1. SCREEN: 70345_screenshot_18.mp4 00:03-00:07.
- 3.13.2. SCREEN: 70345_screenshot_18.mp4 00:07-00:17.
- 3.14. To export the results, click the **Results** tab and right click on the results page [1]. Select **Export to File** and save the results [2].
- 3.14.1. SCREEN: 70345_screenshot_19.mp4 00:05-00:12.
- 3.14.2. SCREEN: 70345_screenshot_19.mp4 00:12-00:28.

4. Geometric Morphometric Analysis Using PAST Software

- 4.1. In **Project Tree**, select **Canonical Variate Analysis**, then **Scores [1]**. Click **File**, choose **Export dataset**, select the data type and **Genotype and Treatment [2]**. Save the CVA scores as a text file **[3]**.
 - 4.1.1. SCREEN: 70345_screenshot_20.mp4 00:03-00:09.
 - 4.1.2. SCREEN: 70345_screenshot_20.mp4 00:09-00:27.
 - 4.1.3. SCREEN: 70345_screenshot_20.mp4 00:28-00:29.
- 4.2. To prepare the file for PAST software open the saved CVA scores in a text editor **[1]**. Replace the term **ID** in the top left corner with **Label** and save the edited file **[2]**.
 - 4.2.1. SCREEN: 70345_screenshot_21.mp4 00:02-00:04.
 - 4.2.2. SCREEN: 70345_screenshot_21.mp4 00:04-00:12.
- 4.3. Open the PAST software and click **File**, then **Open [1]** to select the edited CVA scores file **[2]**. In the import window, select **Names, Data for row and column**, and **Tab** as the separator, then click **Import [3]**.
 - 4.3.1. SCREEN: 70345_screenshot_22.mp4 00:10-00:16.
 - 4.3.2. SCREEN: 70345_screenshot_22.mp4 00:29-00:43
 - 4.3.3. SCREEN: 70345_screenshot_22.mp4 00:43-00:56.
- 4.4. Under the **Show** tab, select **Column attributes [1]**. In the dropdown menu next to **Type**, assign **Group** to the first column containing classifier variables **[2]**.
 - 4.4.1. SCREEN: 70345_screenshot_23.mp4 00:10-00:15.
 - 4.4.2. SCREEN: 70345_screenshot_23.mp4 00:20-00:24.
- 4.5. Highlight the principal component or canonical variate data columns **[1]**. Select **Multivariate**, then **Tests**, and choose **Multivariate normality** to run the normality test separately for principal component and canonical variate data **[2]**.
 - 4.5.1. SCREEN: 70345_screenshot_24.mp4 00:05-00:06.
 - 4.5.2. SCREEN: 70345_screenshot_24.mp4 00:06-00:12.
- 4.6. Click the gray empty cell in the top left corner above **Type** to select the entire dataset **[1]**. Select **Multivariate**, then **Tests**, and choose **Multivariate Analysis of Variance** to perform the analysis **[2]**. Export the MANOVA results to the previously created spreadsheet file **[3]**.
 - 4.6.1. SCREEN: 70345_screenshot_25.mp4 00:03-00:06.
 - 4.6.2. SCREEN: 70345_screenshot_25.mp4 00:06-00:15.
 - 4.6.3. SCREEN: 70345_screenshot_25.mp4 00:22-00:45.

Results

5. Results

5.1. Images of the viscerocranium were acquired for each larva in each genotype and treatment group [1]. Cartilages of the viscerocranium were labeled in a representative image [2], and landmarks were placed on each image to generate landmark-labeled datasets [3].

5.1.1. LAB MEDIA: Figure 1A–D. *Video editor: Sequentially highlight images A to D*

5.1.2. LAB MEDIA: Figure 1E. *Video editor: Zoom in on the labeled cartilage regions in the representative image.*

5.1.3. LAB MEDIA: Figure 1E'. *Video editor: Highlight the numbered black dots marking the landmarks.*

5.2. Principal component 1 accounted for approximately 34% of total shape variation [1], and Principal component 2 accounted for 20% of total shape variation [2].

5.2.1. LAB MEDIA: Figure 3. *Video editor: Highlight the bar labeled “1” showing approximately 34% variation.*

5.2.2. LAB MEDIA: Figure 3. *Video editor: Highlight the bar labeled “2” showing 20% variation.*

5.3. Each Principal component showed specific variation in viscerocranial shape relative to the average shape of all viscerocrania [1].

5.3.1. LAB MEDIA: Figure 2. *Video editor: Emphasize the pink wireframe shape and the black wireframe overlay to show the twisting difference.*

5.4. The principal component analysis plot showed overlapping means among the genotype and treatment groups with no distinct clustering [1]. Ethanol-treated wild-type larvae exhibited a large 95% confidence ellipse of the mean due to the low sample size [2].

5.4.1. LAB MEDIA: Figure 2. *Video editor: Highlight the dots corresponding to untreated and then treated groups.*

5.4.2. LAB MEDIA: Figure 2. *Video editor: Highlight the large black ellipse corresponding to “Wild-type Ethanol.”*

5.5. Canonical variate 1 represented a subtle shortening or lengthening of the ceratohyal joint in the magenta wireframe relative to the average black wireframe [1].

5.5.1. LAB MEDIA: Figure 4. *Video editor: Emphasize the magenta wireframe and the*

black wireframe.

- 5.6. Canonical variate 2 showed a shift medially only on one side of the viscerocranium at the joints between the Meckel's/palatoquadrate and the palatoquadrate/ceratohyal [1].

5.6.1. LAB MEDIA: Figure 4. *Video editor: Highlight the magenta wireframe at the specified joint regions on one side and compare it to the black wireframe.*

- 5.7. Ethanol-treated wild-type larvae exhibited a large 95% confidence ellipse of the mean that overlapped all other groups [1].

5.7.1. LAB MEDIA: Figure 4. *Video editor: Highlight the large black ellipse corresponding to "Wild-type Ethanol" overlapping the other ellipses.*

- 5.8. Multivariate analysis of variance revealed a significant overall effect of genotype and treatment [1].

5.8.1. LAB MEDIA: Table 1.

1. Meckel's
Pronunciation link: <https://www.howtopronounce.com/meckel-s>
IPA: /m'ɛkəlz/
Phonetic Spelling: mek·uhlz
2. palatoquadrate
Pronunciation link: <https://www.howtopronounce.com/palatoquadrate>
IPA: /p'æ.lətɑː.k.wəd.ɪɪt/
Phonetic Spelling: pa·la·tahk·wuhd·rayt
3. ceratohyal
Pronunciation link: <https://www.howtopronounce.com/ceratohyal>
IPA: /s'ɛrɪtoʊ...ɪəl/
Phonetic Spelling: ser·uh·toh·ee·uhl
4. hyomandibular
Pronunciation link: <https://www.howtopronounce.com/hyomandibular>
IPA: /haɪ...əmə.nd'ɪbjʊlə/
Phonetic Spelling: hye·uh·man·dib·yuh·ler
5. morphometric
Pronunciation link: <https://www.howtopronounce.com/morphometric>
IPA: /mɔːrfo..ʊmə'tɪk/
Phonetic Spelling: maw·rfoh·uh·met·rik
6. covariance
Pronunciation link: <https://www.howtopronounce.com/covariance>

- IPA: /kəʊ've(ə)rɪəns/
Phonetic Spelling: koh·vair·ee·uhns
7. wireframe
Pronunciation link: <https://www.howtopronounce.com/wireframe>
IPA: /w'aɪ...əf.ɪəm/
Phonetic Spelling: wye·erf·raym
8. genotype
Pronunciation link: <https://www.howtopronounce.com/genotype>
IPA: /dʒ'ɛnə.ta.ɪp/
Phonetic Spelling: jen·uh·type
9. canonical
Pronunciation link: <https://www.howtopronounce.com/canonical>
IPA: /kən'a:.n.ɪkəl/
Phonetic Spelling: kuh·nah·ni·kuhl
10. variate
Pronunciation link: <https://www.howtopronounce.com/variant>
IPA: /v'ɛɪ.e.ɪt/
Phonetic Spelling: vair·ee·ayt
11. multivariate
Pronunciation link: <https://www.howtopronounce.com/multivariate>
IPA: /mʌltɪ'veəriət/
Phonetic Spelling: muhl·tee·vair·ee·uht
12. ethanol
Pronunciation link: <https://www.howtopronounce.com/ethanol>
IPA: /'ɛθən.ɑ:l/
Phonetic Spelling: eth·uh·nahl
13. ellipse
Pronunciation link: <https://www.howtopronounce.com/ellipse>
IPA: /ɪ.l'ɪps/
Phonetic Spelling: ih·lips
14. viscerocranium
Pronunciation link: <https://www.howtopronounce.com/viscerocranium>
IPA: /vɪ.s.ə.ɪk..'.ɪɛniəm/
Phonetic Spelling: vih·ser·ruh·kray·nee·uhm