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A comprehensive method to assess D. melanogaster's viability from embryo to adult

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Dear Stephanie Ray Weldon, Editor of JoVe,

We are happy to submit to JoVe a method developed in our lab that accurately tracks viability of Drosophila from embryo to adult. Albeit simple and straightforward, we have not found any published protocol that addresses how to thoroughly follow viability of a cohort of F1 siblings as they develop in food vials. By combining Chi square analyses and direct transfer of grape-agar discs containing embryos/L1 larvae onto colored food in clear vials, it is possible to assess viability at each developmental stage without having to wash embryos off the plate to transfer to vials, a common source of technical errors that confound results. This method can be combined with the use of NIGHTSEA™ Fluorescence Viewing Systems when using alleles that are marked with fluorescent moieties.

We have produced a high-resolution video of which a low-resolution version accompanies this submission.

We believe that our work submitted to JoVe titled "A comprehensive method to assess *D. melanogaster*'s viability from embryo to adult" will prove very useful to the Drosophila research and teaching community.

We appreciate your invitation to submit and hope that our work can be published in JoVe.

We thank you in advance for your time and effort in evaluating our work.

Best regards,

Cintia F. Hongay, PhD.

TITLE:

A Direct and Simple Method to Assess *Drosophila melanogaster*'s Viability from Embryo to Adult

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KEYWORDS:

Drosophila melanogaster, drosophila life cycle, embryo survival, adult viability, embryo hatching, L1/L2, L3 to prepupae, pupae to adult viability assessment

SUMMARY:

This protocol is designed to assess the viability of *Drosophila* at every developmental stage, from embryo to adult. The method can be used to determine and compare the viability of different genotypes or growth conditions.

ABSTRACT:

In *Drosophila melanogaster*, viability assays are used to determine the fitness of certain genetic backgrounds. Allelic variations can result in partial or complete loss of viability at different stages of development. Our lab has developed a method to assess viability in *Drosophila* from embryo to fully mature adult. The method relies on quantifying the number of progeny present at different stages during development, starting with hatched embryos. After embryos have been quantified, additional stages are counted, including L1/L2, pupae, and mature adults. After all stages have been examined, a statistical analysis such as the chi-square test is used to determine if there is a significant difference between the starting number of progeny (hatched embryos) and later stages culminating in the observed number of adults, thus rejecting or accepting the null hypothesis (that the number of hatched embryos will be equal to the number of larvae, pupae, and adults recorded throughout the stages of development). The primary advantage of this assay is its simplicity and accuracy, as it does not require an embryo rinse to transfer them to the food vial, avoiding losses from technical errors. Although the protocol described here does not directly examine L2/L3 larvae, additional steps can be added to account for these. Comparing the number of hatched embryos, L1, pupae, and adults can help determine if viability was compromised during the L2/L3 stages for further studies (the use of colored food helps with visual identification of larvae). Overall, this method can help *Drosophila* researchers and educators determine when viability is compromised during the fly life cycle. Routine assessment of stocks using this assay can prevent accumulation of secondary mutations that may affect the phenotype of the originally isolated mutant, especially if the original mutations affect fitness. For

this reason, our lab maintains multiple copies of each of our *Dm ime4* alleles and routinely checks the purity of each stock with this method in addition to other molecular analyses.

INTRODUCTION:

Lifespan is affected by genetic and non-genetic factors. In standard lab growth conditions at room temperature, our lab has observed significant variation of fitness and viability among different *Dm ime4* alleles grown under identical conditions (**Figure 1** and **Supplemental Figures**). Viability studies are frequently done to investigate the effects of a certain allele combination or growth condition in population genetic studies[1-4]. However, detailed analyses of viability within a non-complementary group of mutations are hard to find in the scientific literature. An allele is usually labeled "non-essential" if the researcher finds a few individuals homozygous for that allele within the food vial that houses the balanced stock[5,6]. However, accurate Chi-square analyses to assess whether these homozygotes arise at the expected Mendelian ratios are not reported[5,6]. The most permissive temperature for any *Drosophila* stock is room temperature (22–23 °C) and, with appropriate nutrients, the life cycle of wild-type flies takes approximately twelve days to complete[7,8]. As the duration of each developmental stage of wild-type *Drosophila* is known[7,8], the method described in this report can be used to examine whether the *Drosophila* strain under study is fit at each stage in comparison to a control appropriate for the genetic background tested. In contrast to studies that focus on one specific aspect of development[9], this protocol provides a practical way to assess viability at different developmental stages.

In our lab, this protocol is used to assess the viability of stocks that are deficient for Drosophila Inducer of Meiosis 4 (*Dm ime4*). *Dm ime4* is an essential gene[10] that encodes an RNA methyltransferase with critical roles in RNA metabolism in *Drosophila* and other multicellular organisms[5,6,10-14]. To quickly evaluate novel alleles of *Dm ime4* generated via CRISPR/Cas9 (**Supplemental Figures**), an end-point viability assay was performed that only counted adult progeny produced within vials of balanced stocks (**Figure 1**). Some of the stocks used were described in previous *Dm ime4* reports[5,6]. Homozygous mutants emerged at sub-Mendelian levels, as determined by chi-square analyses (**Figure 1** and **Supplemental Materials**). To assess whether these lower-than-expected numbers were due to fewer embryos being laid, or fewer hatched embryos, or loss of viability in L1/L2 or pupae, we expanded the tracking to include counts at each of these developmental stages (**Figure 2**, **Figure 3**, **Figure 4**, **Figure 5**, **Figure 6**, **Figure 7**, **Figure 8**, and **Figure 9**).

Here, we describe the method using wild-type (OreR) flies. To empirically test this method for use with other genetic backgrounds or Drosophila species, we recommend using OreR as a reference and adjust timepoints according to the experimental organism. The protocol was further evaluated to assess viability of progeny generated by crossing virgin wild-type females with males from a heterozygous *Dm ime4* mutant stock [5,6] (**Figure 4**).

PROTOCOL:

1. Media preparation

1.1. Prepare grape agar according to manufacturer instructions (see **Table of Materials**) and pour into a 35 mm Petri dish to half-full (**Figure 5**). Allow to solidify for approximately 1 h. After the grape agar solidifies, immediately use or store at 4 °C.

1.2. Gently make scratches across the agar plate using a small plastic knife (flies like to lay on uneven surfaces) leaving the middle of the plate without scratches (**Figure 5**). Place a small amount of yeast paste (made fresh; see **Table of Materials**) at the center of plate (**Figure 5**).

2. Embryo collection mini cage set up

2.1. Set up a cross using two virgin females and one young male inside the embryo collection cage containing the grape agar plate supplemented with yeast paste (**Figure 5**).

2.2. After 24 h, inspect the cages for laid embryos without opening the cage by looking at the bottom of the agar plate. If embryos have been laid, remove the agar plate from the chamber and place it inside a humid chamber for microscopic observation (**Figure 6**). If several days of laying are scored (e.g., fertility/longevity assays), keep the breeding parents and replace the plate with a fresh one prepared as described. Early collections of less than 24 h can be done but, when using virgin females, be aware that they will not lay until 48 h after emerging from their pupa case.

2.3. Cover the agar plate containing embryos with the Petri dish lid to avoid dehydration and place it immediately inside a humid chamber (**Figure 7**). Observe under a dissecting microscope and record hatched embryos and L1. Replace lid and store at room temperature in humid chamber until all embryos had hatched and developed into L1 larvae (**Figure 6**).

3. Counting embryos and larvae

3.1. After 48 h, observe the plates under the dissecting microscope and record the numbers one last time before transfer of agar disc to food vial. Fertilized/viable embryos should become L1 by then. Longer incubation periods prior to transfer are possible but be aware that plates may start losing moisture and the agar may crack compromising a clean transfer to food vials (**Figure 7**). To ensure the plates remain hydrated and embryo viability is not compromised while counting, avoid using a direct gooseneck light over the agar surface (**Figure 7E** shows an appropriate distance).

3.2. After counting, cover the plate and store it in the humid chamber until ready to transfer. Record findings.

4. Transfer the grape agar disc to a food vial to monitor viability during development

4.1. Once the numbers are recorded (hatched embryos/L1/L2), use a spatula to carefully transfer the grape agar disc to a vial large enough to accommodate a 35 mm disc containing Drosophila

food media prepared according to manufacturer's instructions (refer to list of reagents). Place the grape agar disc L1-side down on the food (**Figure 7**). After transferring the agar disc with larvae to the food vial, carefully inspect the empty Petri dish for any larvae left behind (**Figure 7E**).

4.2. Set up a schedule to inspect food vials daily, at roughly the same time each day, to ensure L2/L3 larvae are observed making their way to the food in the vial (**Figure 8**).

4.3. Record the number of pupae and adult Drosophila (**Figure 9**). Keep counting until no more adults are observed and avoid counting the following generation (do not count past 9 days after observing the first adults).

4.4. Observe and record the findings. Adjust the time frame of embryo collection, counting, and recording accordingly to the mutant stock being used.

4.5. Perform a chi square analysis. The null hypothesis assumes 100% viability such that the number of adults will be equal to the number of hatched embryos and L1 originally recorded and transferred to the food vials

REPRESENTATIVE RESULTS:

This method accurately and reproducibly allows one to gauge viability from embryos to emerged adults when coupled to chi square analyses.

In initial studies, after counting embryos and larvae, the grape agar was placed inside the vial upright on the side of the vial. Unfortunately, when the agar was placed on the side of the vial many of the embryos and larvae did not mature to adults. This was likely due to the grape agar disc drying out (**Figure 3**). This placement introduced an environmental variable (hydration of agar disc) that could confound the results. Approximately 39% of the progeny was lost between embryos to adult as only 61% of adults emerged. The biggest losses occurred between the L1 larvae (counted on surface of grape agar plates) and pupae (counted on walls of food vials) counts. However, when the grape agar was placed faced down inside the vial in direct contact with the food surface, less than 6% of the progeny was lost (**Figure 2**). The agar remained hydrated as the entire grape agar disc remained in contact with the moisture of the instant food inside the vial (**Figure 7**, **Figure 8**). These results indicate that the position of the agar inside the vial is important for obtaining reliable data and minimize contribution of environmental variables. Further data to support the effectiveness of the method was collected by comparing wild-type progeny used in initial method validation and progeny produced by crossing virgin wild-type females to males from a balanced *Dm ime4* mutant stock (**Supplemental Figure 2**, *ime4Δnull/TM3*^{sb}). Approximately 91% of the progeny matured to adulthood compared to 94% of the progeny from wildtype (**Figure 4**). This difference was not statistically significant.

Homozygous *Dm ime4* mutant males[5] were also used to provide further validation of the method. However, the homozygous mutants were too sick to reproduce and died before embryos were

deposited. Therefore, one limitation of the experiment is that males need to be healthy enough to reproduce to generate a starting embryo population to track.

FIGURE AND TABLE LEGENDS:

Figure 1: *Dm ime4* mutant stocks emerge at sub-Mendelian levels. Stocks in which portions of the catalytic domain or Ado-Met binding domain were mutated are shown (refer to **Supplemental Figure 1** for details). Some stocks had portions of either domain replaced with Ala (alanine-scanning mutagenesis), while other stocks had either or both domains deleted. The ratio of observed number of homozygotes to the expected numbers is represented in percentages (compared to heterozygous sibling controls, refer to **Supplemental Figure 3** for sorting scheme and chi square analysis). Chi square analyses were performed to determine if the difference between observed and expected was statistically significant and not due to chance alone (p-values <0.01). Error bars represent standard error of the mean (minimum of three trials per cross indicated). A spreadsheet with all the data can be found in **Supplemental Materials**.

Figure 2: Agar position inside the vial can introduce unintended environmental variables. Histogram of the number of individuals at different stages of development counted from grape agar disc embryo side down in food vial or agar on the side of the food vial. Placing the agar disc embryo-side down in the vial resulted in 94% (SD ± 0.02) of initial embryos surviving until adulthood. Placing the agar on the side of the food vial resulted in only 61% (SD ± 0.07) of the originally counted embryos reaching adulthood. A spreadsheet with all the data can be found in the **Supplemental Materials**. The expected numbers at each stage is set as the initial number of embryos/L1 counted on the grape agar disc prior to transfer to food vial (Null hypothesis: 100% of hatched embryos develop). The observed numbers are the actual number of individuals counted for the developmental stage indicated. Thus, each stage is compared to the initial number of hatched embryos counted on the grape agar plate. Chi square analyses were performed to determine if the difference between observed and expected was statistically significant and not due to chance alone using a p-value threshold of 0.05. The difference was significant for embryos grown with agar on the side of the food vial, but not for those grown in a disc embryo side down. Errors bars represent standard error (minimum of three trials per cross indicated).

Figure 3. Cartoon showing two ways grape agar discs carrying embryos/L1s can be placed inside the food vial. There was a significant difference between the total number of embryos and adult progeny based on the position of the agar in the food vial. The difference stems from an environmental variable (hydration) created by the difference in disc placement inside the food vial: there is a statistically significant difference between expected and observed numbers in the larvae and pupae counts when the agar disc was placed on the side of the vial versus no significant difference in expected versus observed numbers when the agar disc was in contact with the food.

Figure 4. Histogram showing the number of individuals at different stages of development from wild-type males versus males from a *Dm ime4* mutant balanced stock. Balanced males

crossed to virgin wild-type females as described in 2.1. Agar was transferred embryo-side down into food vials. The average ratio of adults emerging from the original embryos counted in grape agar discs from wild-type X wild-type was 94%, SD ± 0.02, whilst that ratio for *Dm ime4* mutant/+ X wild-type was 91%, SD ± 0.01. Statistical analysis was performed as described for **Figure 2**; differences were not significant for either group.

Figure 5: Experimental setup. (**A**) Embryo collection mini-cages and grape-agar mini-Petri dishes. Plates are placed inside a humid chamber (standard Petri dish with absorbent paper disc saturated with water) to prevent dehydration. A small amount of yeast paste was placed at the center of each grape-agar plate. (**B**) Detach the embryo collection plate holder lid (red) and place a prepared plate, yeast side up. (**C**) Transfer the cross to the embryo cage and immediately place a Petri dish lid to hold flies inside. (**D**) Quickly remove cover and flip the embryo cage so opening contacts grape-agar plate. Incubate for 24–48 h, inspecting daily.

Figure 6. Embryo development on grape-agar plates. (**A**) Wildtype embryos deposited on the grape-agar mini-Petri dishes are counted under a dissecting microscope. Keep plates inside humid chamber when not visualizing and avoid direct light to prevent dehydration. (**B**) Grape-agar plates are kept in a humid chamber and hatched wildtype embryos are recorded. This photo shows three wildtype L1 larvae that developed from embryos shown in A. (**C**) Example of recorded numbers of embryos (day 1) and L1 larvae (day 2) for the two plates shown in **Figure 5**. The "expected number" for the null hypothesis is determined by the number of embryos that hatched and became L1 larvae at the time of transfer to the food vial (table below). For wildtype flies, almost all embryos hatch and develop into L1 larvae. This may not be the case for other genetic backgrounds and this method is used to determine these differences in viability.

Figure 7. Transferring larvae. (**A**) After recording L1 counts, the grape-agar discs are carefully removed from the Petri dish using a clean (ethanol-wiped) spatula (**B**,**C**) and transferred L1 side-down to contact the food in the vials as shown in **D**. (**E**) The empty Petri dish is carefully inspected to detect any larvae left behind. If larvaee left behind are alive and can be transferred to the food vial, do so carefully and immediately (**F**). If larvae left behind are dead or cannot be transferred, record this fact to correct the "expected number" for subsequent calculations.

Figure 8. L2 larvae make their way into the food. See crevices and grooves between the grape-agar disc and the blue food. Set up a schedule to observe the vials daily, preferably at the same time of day.

Figure 9. Adult emergence. (**A**) L3 larvae make their way out of the food to become pupae. (**B**) Adults start to emerge on day 11. Set up a schedule to observe the vials daily, preferably at the same time of day and carefully record your observations. Stop counting when all the pupae have emerged (count empty pupae cases) and avoid counting the next generation of flies by stopping the experiment on day 15 and count dead pupae if any (black/dried pupae). Mutants with lengthier life cycles may need longer periods of time, which need to be determined empirically.

DISCUSSION:

In summary, this method provides an accurate and simple assessment of viability in *Drosophila*. The entire protocol takes approximately 14 days to complete. The procedure does not require expert technical skills; however, proper timing, a schedule of daily observations, and careful agar transferring is important for accuracy and reproducibility.

In addition to the placement of the grape-agar disc embryo-side down in the food vial, another crucial step in the procedure is transferring the agar disc to a food vial no later than 48 h after removing the grape agar plate from the embryo collection mini cages. Transferring the agar after 48 h resulted in embryo and larvae loss, likely due to dehydration of the agar disc. To count L2/L3 transitions, the plate needs to incubate for 72 h. If this stage is crucial, grape agar plates should be poured thicker and a humid chamber must be used to prevent desiccation. The crosses were set up in embryo collection cages that accommodate 35 mm plates; however, this procedure can be performed using larger embryo collection cages as well, such as one that accommodates 60 mm or 100 mm Petri plates. Food bottles must then be big enough to accommodate those sizes.

There are steps that can be added to this protocol. As mentioned above, L2/L3 transitions are challenging to quantify on plates due to the time required and the potential dehydration of the agar. Additionally, larvae become highly mobile on the surface of the agar, posing a challenge to accurately count them. Placing the plates in the refrigerator for 30 minutes or adding a few drops of a mild anesthetic (lidocaine solution) on the surface of the agar prior to counting L2/L3 larvae can help slow down their movements to count them more accurately. A caveat to these modifications is that they can introduce variables (cold sensitivity, anesthetic sensitivity) and confound the viability results. Even without these steps, by using colored food, researchers can quantify wandering L3s/prepupae as they settle on the side of the food vials to initiate pupation. A limitation to this method is that it requires the adults used in the crosses to survive being anesthetized with CO_2 for phenotyping to set up the crosses in the embryo collection cages. *Dm ime4* homozygous mutant males do not recover well and die a few hours after waking up from CO_2 treatment. Other methods to immobilize adults for sorting can be explored, such as placing vials in the refrigerator for a few minutes and then transfer vials to crushed ice to slow movement and sort quickly and efficiently.

Apart from comparing allelic strengths and their effects on viability, this method can be used to screen sensitivity or resistance to defined pharmaceutical compounds. Unlike other methods that screen compound toxicity in cell culture[15-18], this method uses whole organisms, making assessment of developmental effects easier to analyze. In sum, using this protocol and modifications therein, will allow to measure allelic strengths as well as the effects of environmental factors and chemical compounds on *Drosophila* viability, fecundity, fertility, lifespan, and duration of developmental cycle.

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DISCLOSURES:

There are no conflicts of interest.

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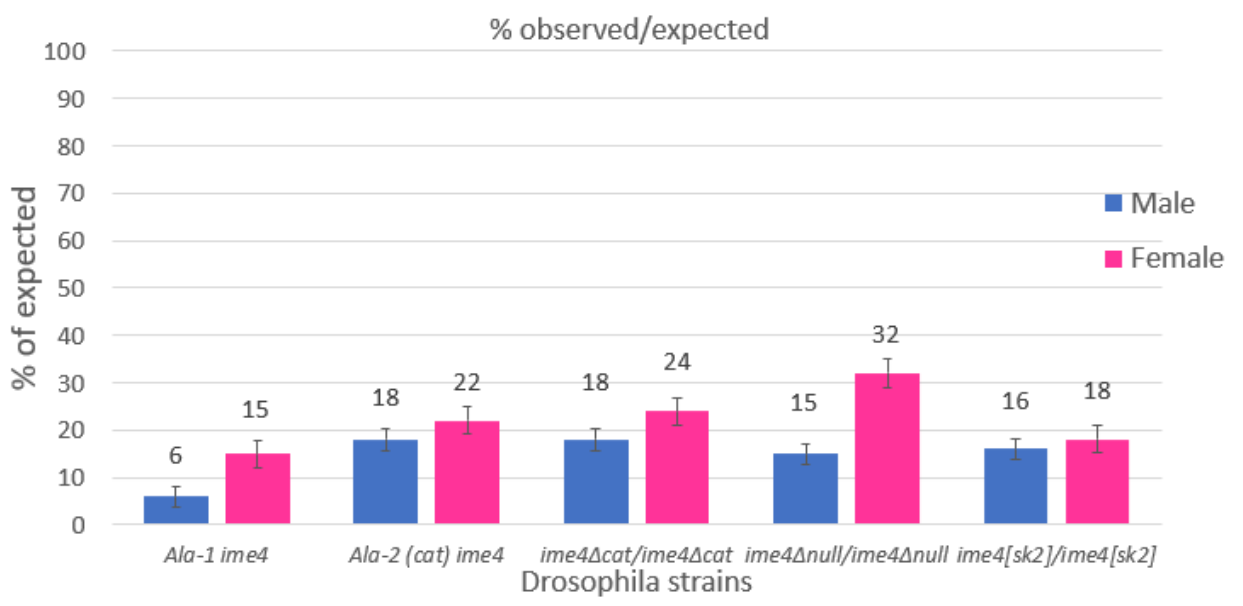
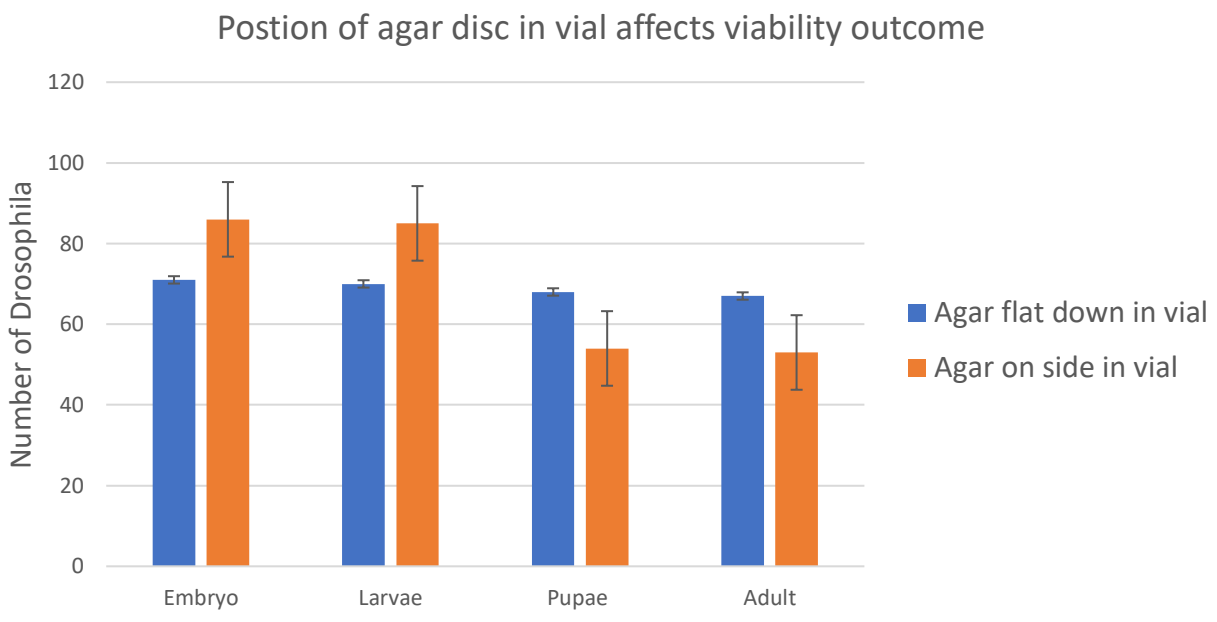
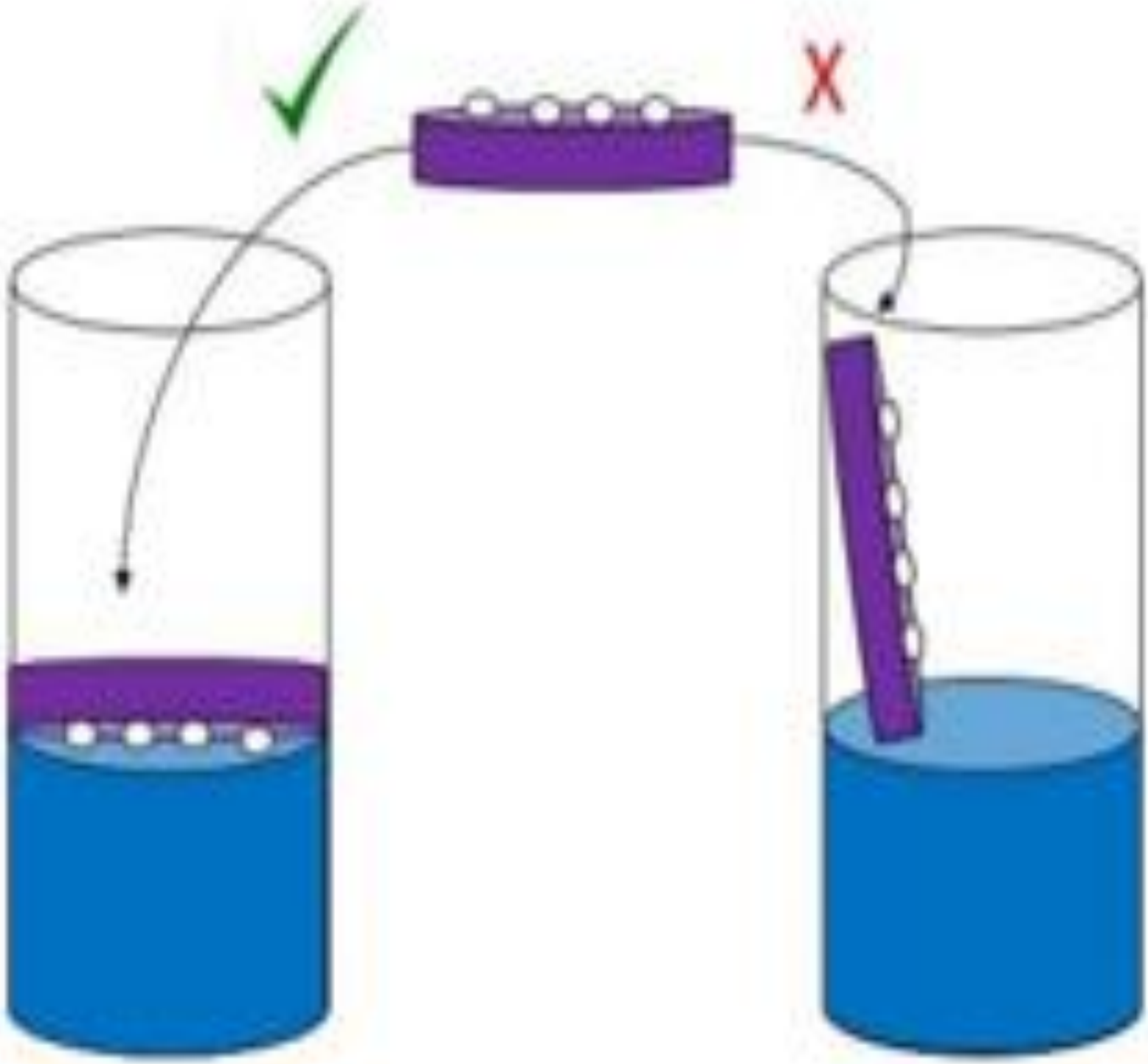
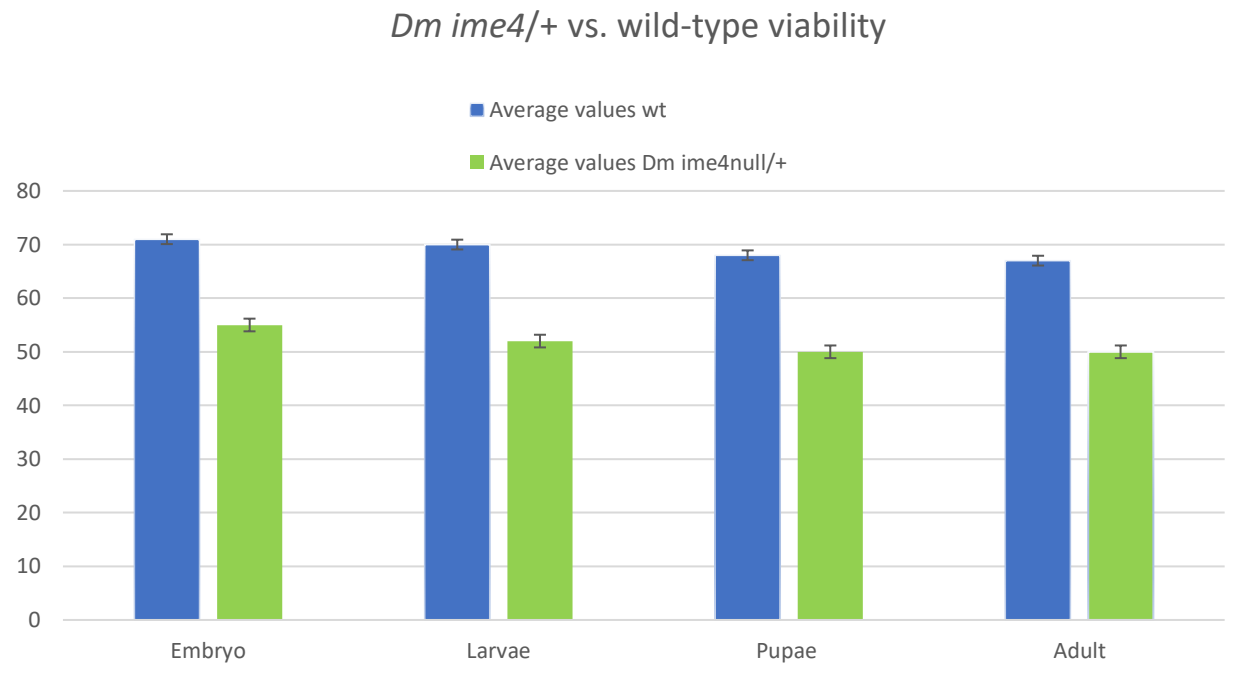
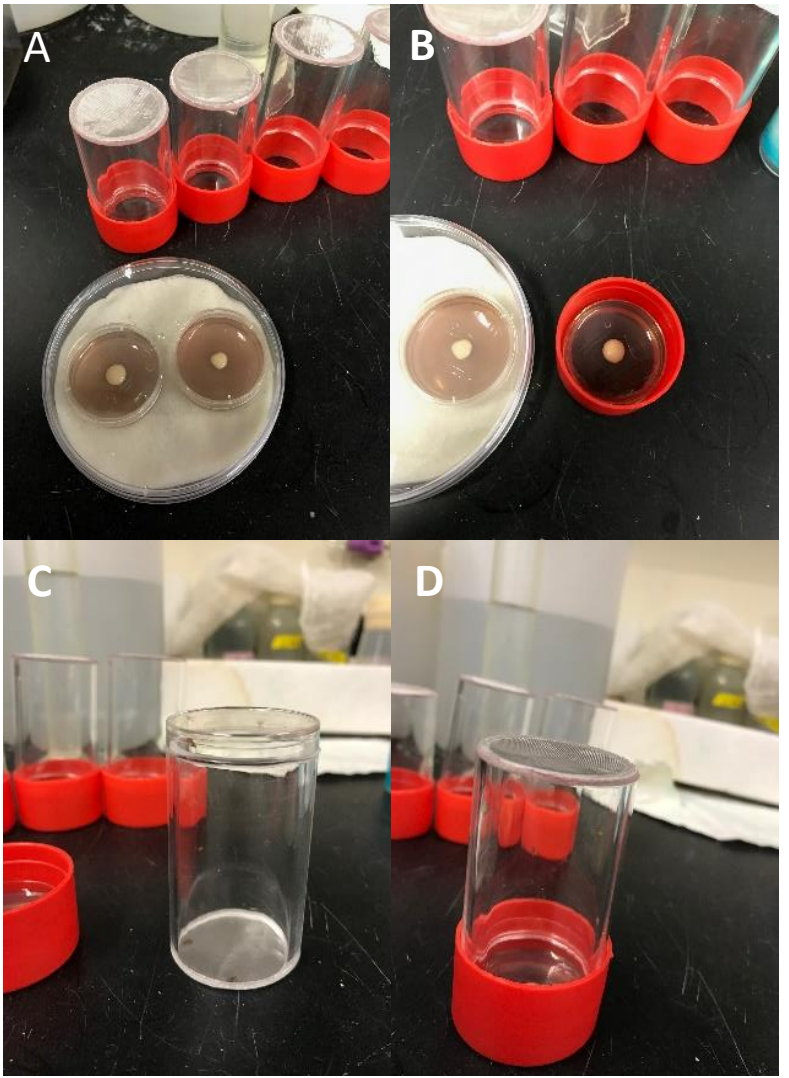
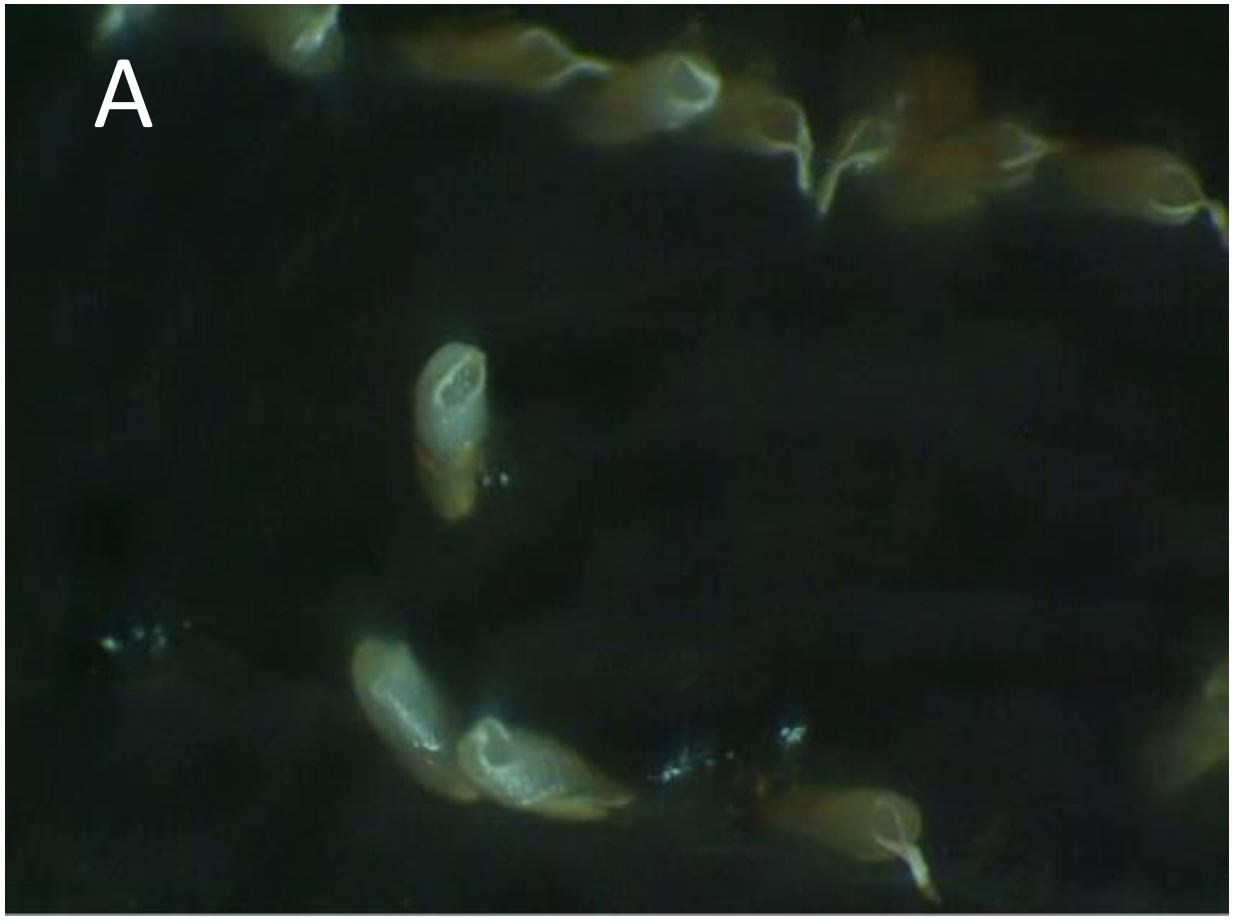

% observed/expected
100
90
80
70
60
50
40
30
20
10
0
% of expected
Male
Female
6
15
18
22
18
24
15
32
16
18
Ala-1 ime4
Ala-2 (cat) ime4
ime4Δcat/ime4Δcat
ime4Δnull/ime4Δnull
ime4[sk2]/ime4[sk2]
Drosophila strains

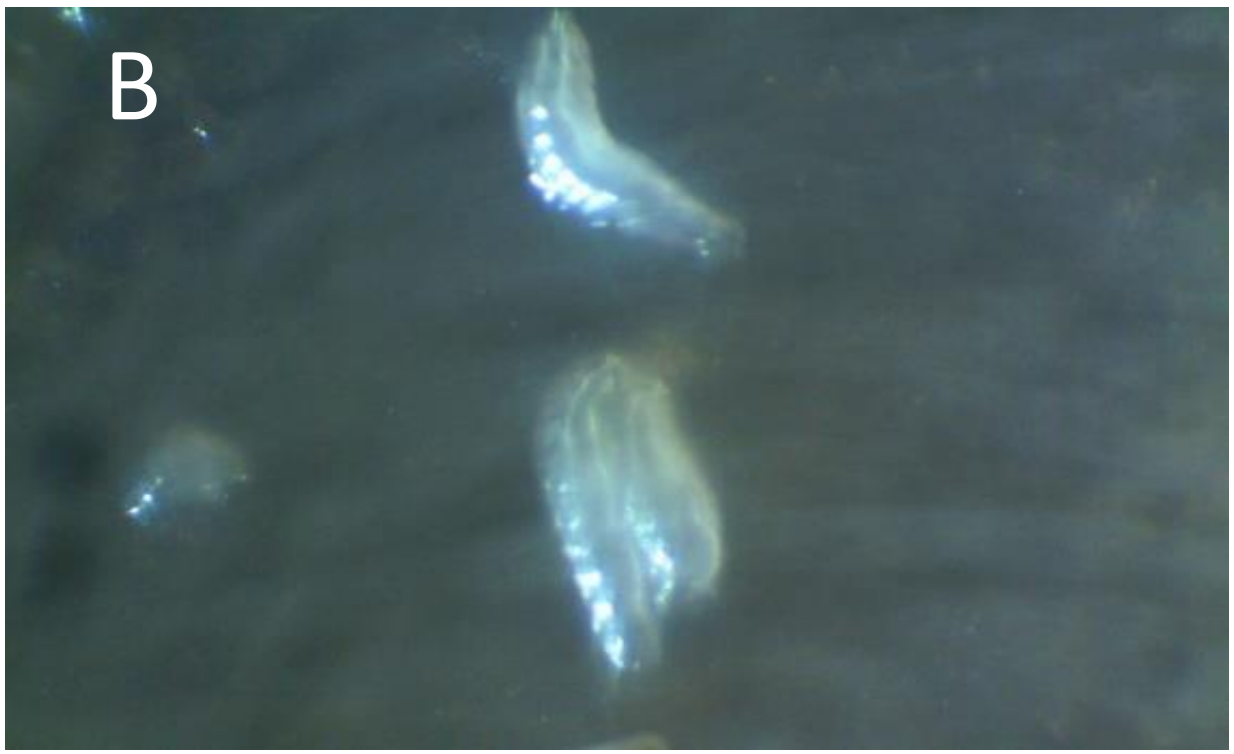

Postion of agar disc in vial affects viability outcome
Number of Drosophila
120
100
80
60
40
20
0
Embryo
Larvae
Pupae
Adult
Agar flat down in vial
Agar on side in vial




Dm ime4/+ vs. wild-type viability
Average values wt
Average values Dm ime4null/+
80
70
60
50
40
30
20
10
0
Embryo
Larvae
Pupae
Adult

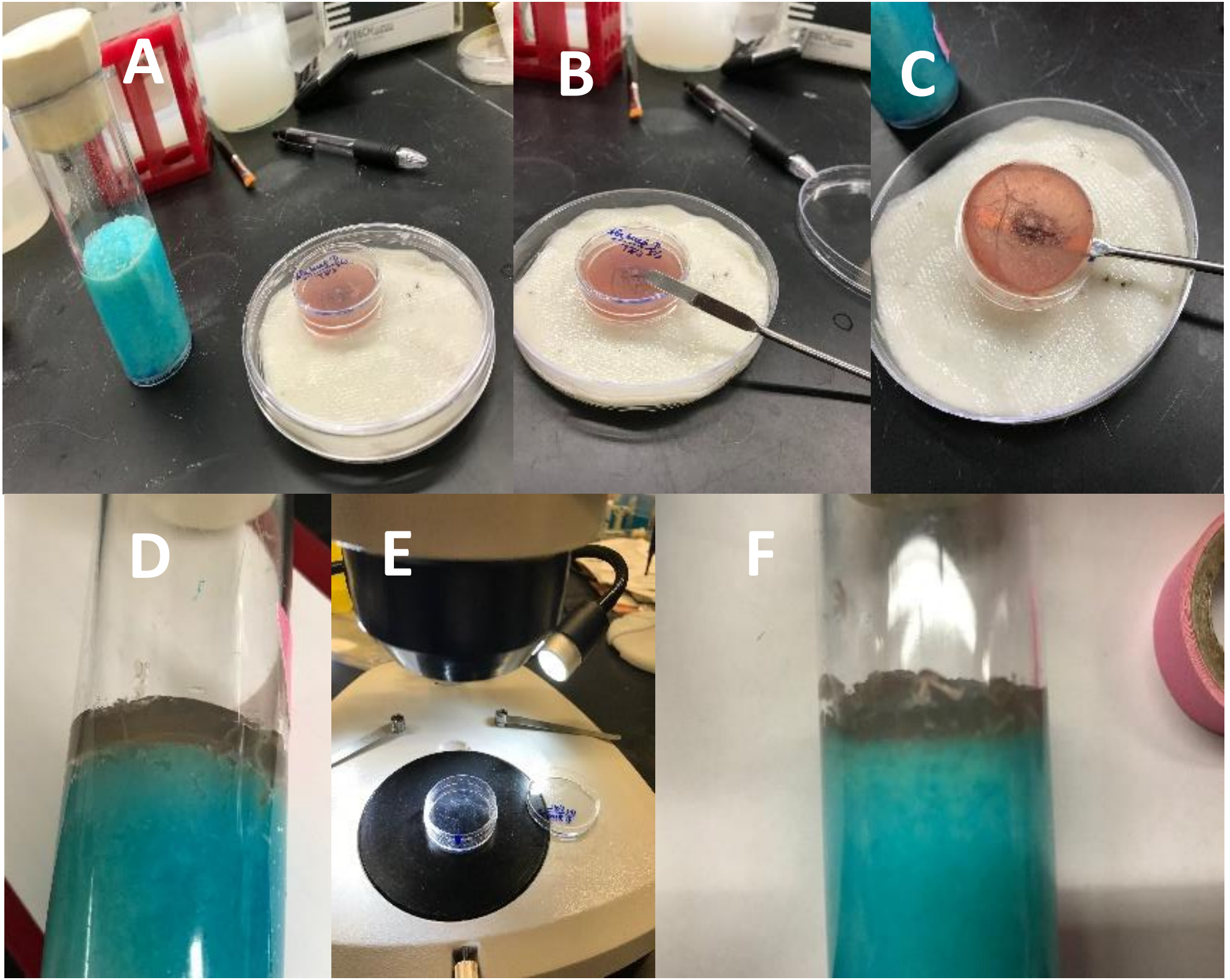

A
B
C
D





C.

	Embryos	L1	
Plate 1	64	62	Expected number=62
Plate 2	69	69	Expected number=69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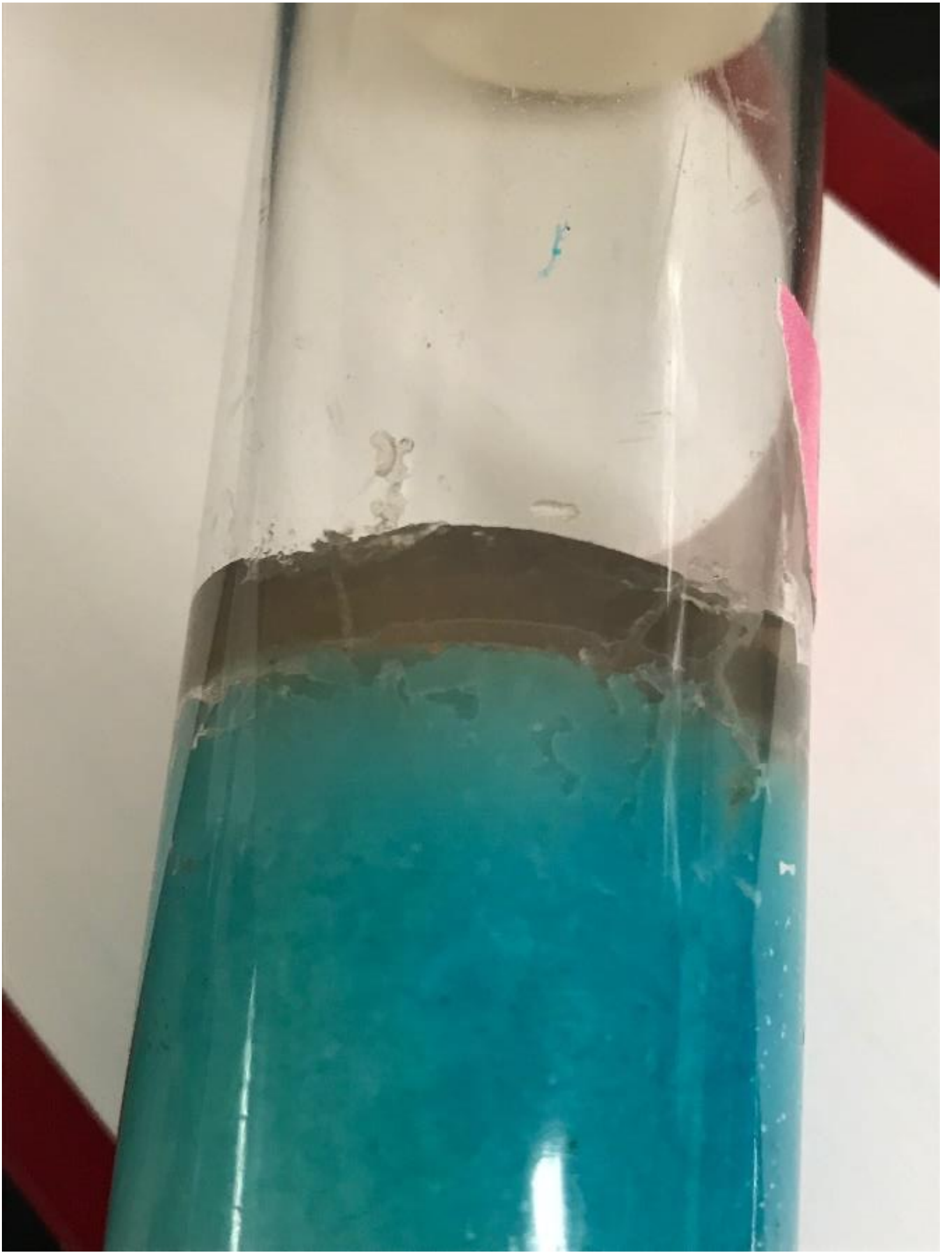

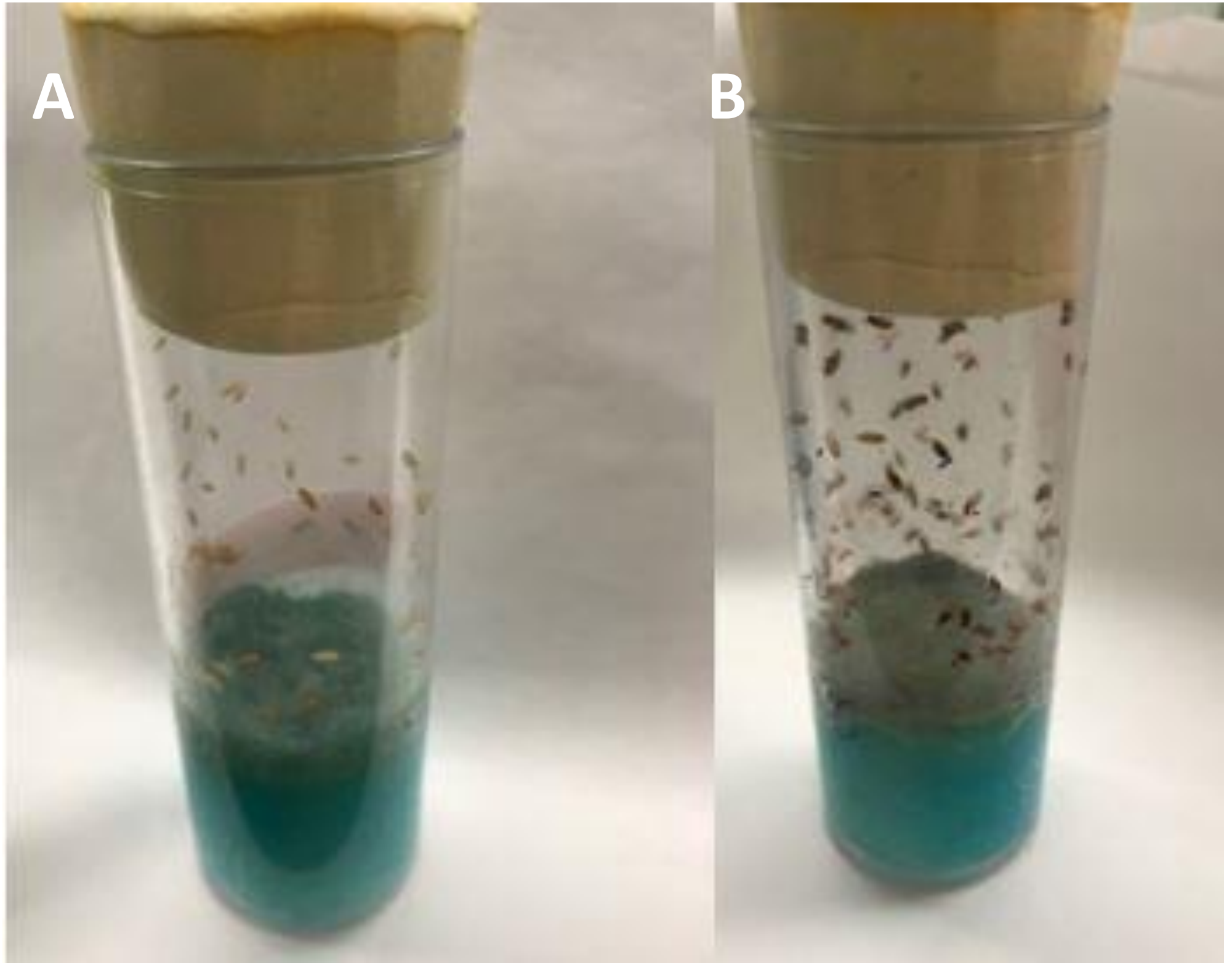
A
B

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Antimold additive	Carolina		
Beaker	Fisher Scentific	S76100J	Beaker
Benchmark scientific digital hotplate	The Lab Depot	H3760H	Hot plate
Britta-filtered tap water to prepare grape agar and instant fly food	Any tap water filtration system		
CO2 pistol or FlyNap	Carolina		Anesthetic to sort/count adult flies
Dissecting microscope/stereoscope	Amscope	SM-1TSZ-V203	Dissecting microscope
Drosophila culture vials and stoppers	Carolina	173120	Food vials for growing Drosophila
Embryo collection mini cage	Genesee Scientific	59-105	chamber used to gather embryos
Erlenmeyer flask	Sigma-Aldrich	70980	Erlenmeyer flask
Formula 4-24 Instant Drosophila Medium, Blue	Carolina		https://www.carolina.com/drosophila-fruit-fly-g
Grape agar	Genesee Scientific	47-102	Media used for agar plates
Instant fly flood	Carolina	173210	Blue media for food vials
Large Petri dish or any clear container to be used as a humid chamber by placing wet paper towels to keep the grape-agar plates			
Metal spatula (small)	Carolina		
Microwave	Any		To cook grape agar
Petri dish	Kord-Valmark	2901	Petri dish for grape agar
Stir bar	The Lab Depot	58948-981-EA	Magnetic stir bar

enetics/formula-4-24-drosophila-medium-blue/FAM_173210.pr

moist during incubation/pre and post microscopic observation

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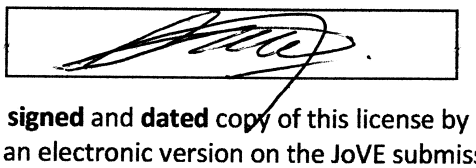
Signature:	
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Editorial comments

1) The manuscript was proofread.
2) Additional keywords/ phrase added to bring the total to six.
3) Summary shortened to less than 50 words. The protocol and applications are described in the protocol.
4) The abstract was revised to describe the method in more general terms. Also, the purpose of the method, advantages and limitations are stated in the abstract and discussion.
5) All commercial language has been removed.
6) Personal pronouns have been removed from the protocol.
7) Protocol has been revised to contain action steps only. Text that could not be in imperative tense was added as note.
8) Protocol has been revised to contain no more than 3 action steps and no more than 4 sentences.
9) Weblink has been removed and reference has been inserted.
10) Limitations of technique has been added.
11) Figure 2 has been combined into one image file. Figure 2 contains two histograms and a cartoon.
12) Trademarks have been removed. (Needs to be removed from table of authors).

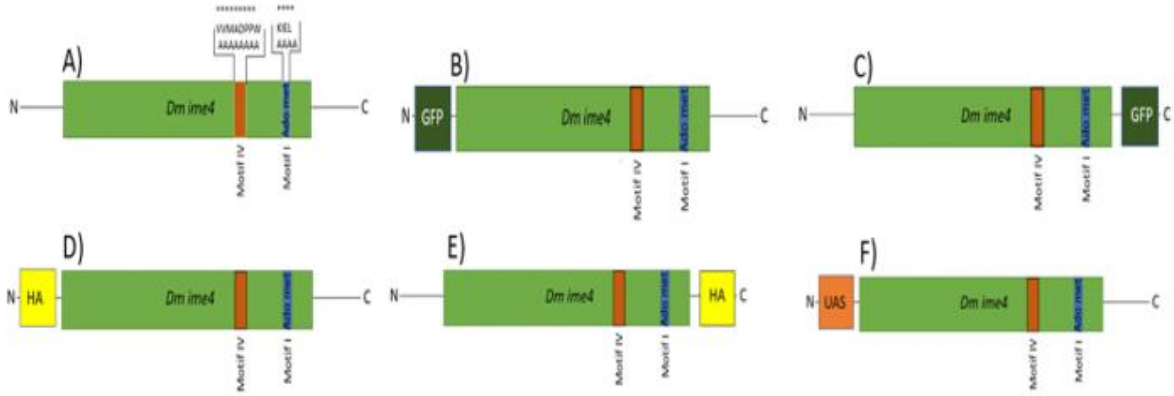
Reviewer 1 comments.

1) Personal pronouns such as we have been removed from the text.
2) *Dm ime4* full name and function has been added to the text.
3) Homozygotes have been substituted for homozygous line 67.
4) Commercial names such as Genetivision has been removed.
5) Described has been removed line 86.
6) The p-values have been added to the figure legends. Original excel files will be sent along as Supplemental Materials.
7) The percentage of embryos that developed to adulthood along with standard deviation have been added to the legend for Figure 2. Also appearing in the excel sheet.

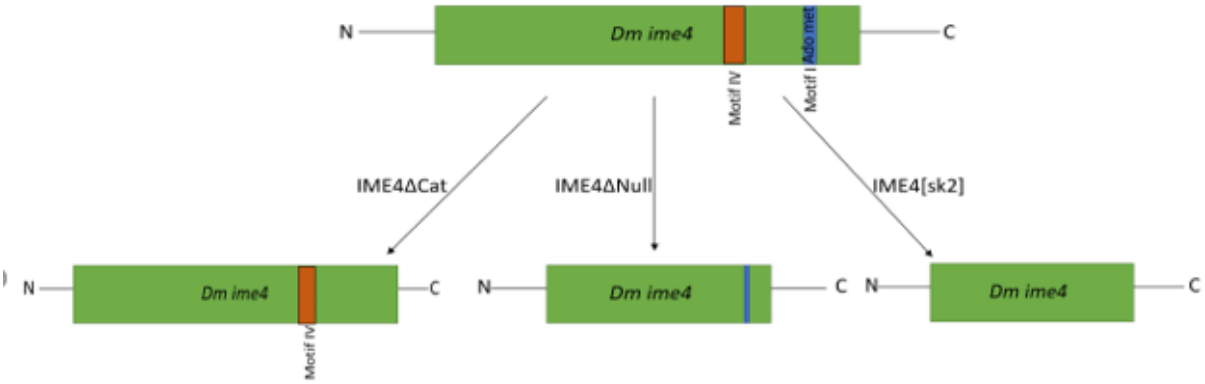
Reviewer 2 comments

1) Each experiment was performed a minimum of 3 times, independent biological replicates were used to ensure reproducibility. Each experimental cross included two virgin wildtype females and 1 wildtype male.
2) A Chi square test was used to determine if there was a significant difference between the two different grape agar positions. The p-values have been added to the legend of Figure 2. Also highlighted in yellow in the excel sheet used in all calculations and bar graphs.
3) A Chi square test was used to determine the viability phenotype of *Dm ime4* mutants, the p-values have been added to the figure legend.
4) The error bar represents the actual numbers of the counts at individual stages for each biological replicate. The variance in the error bars are a result of different starting numbers of progeny per trial, not the percentages of progeny lost. However, the statistical analysis does suggest a significant difference between the agar positions. Please refer to the excel sheet sent as Supplemental Materials.
5) Although we did not examine L3, L3 larvae can be quantified as they crawl up the side of the vial. The L3 quantification statement has been added to the discussion (Line 177).

6) This is a valid point that it would be beneficial to look at viability of different genotypes and phenotypes; however, we used wildtype OreR to develop the reported assay and for proof of principle and troubleshooting. Thus wildtype Ore-R is used to provide a baseline for future experiments



Supplemental Figure 1. Mutations in *Dm ime4* generated by CRISPR gene editing. A) Alanine amino acid substitutions in motif I and motif IV. B) Addition of GFP tag on the N-terminal end. C) Addition of GFP tag on the C-terminal end. D) Addition of HA tag on the N-terminal end. E) Addition of HA tag on the C-terminal end F) Addition of UAS construct at the N-terminal end.



Supplemental Figure 2. Deletions in *Dm ime4* generated by CRISPR gene editing. Three different deletion alleles are shown *ime4ΔCat* (catalytic domain deleted), *ime4ΔNull* (SAM binding domain and portions of catalytic domain deleted) and *ime4[sk2]* (catalytic domain and SAM binding domain deleted).

Dm ime4 **mutant/TM3^{Sb} X** ***Dm ime4*** **mutant/TM3^{Sb}**

1/4 ~~*Dm ime4* Homozygous~~	1/4 ***Dm ime4*** $^{+/-}$ **Heterozygous (Stubble)**
1/4 ***Dm ime4*** $^{+/-}$ **Heterozygous (Stubble)**	1/4 ***Dm ime4*** $^{-/-}$ **Homozygous (non-Stubble)**

But TM3^{Sb}/TM3^{Sb}
Homozygous balancer
Dies before adulthood

Stubble

The corrected ratios are 2/3 heterozygous, 1/3 homozygous *Dm ime4* mutants

Adults: 1/3 expected to be *Dm ime4* $^{-/-}$ Homozygous.
E.g. **out of 120 adults, 40 are expected to be homozygous *Dm ime4* mutant.**

Chi-square Goodness of Fit Test Formulas

Null Hypothesis → $H_0 : Observed = Expected$

Alternate Hypothesis → $H_a : Observed \neq Expected$

The null and alternate hypotheses are always the same with a Goodness of Fit Test.

Test Statistic (that symbol is called "Chi-squared") → $\chi^2 = \sum \frac{(O-E)^2}{E}$

$df = (\#\ of\ categories) - 1$

$P - Value = \chi^2 cdf(\chi^2, 9999, df)$

O is the observed count for each category and E is the expected count for each category.

Instead of a normal or t distribution, we now have a chi-squared distribution

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Strain	Line 1-3	Ala1-IME4/tm3		homozygotes	
Drosophila gender		male	female	male	female
1) Number counted		17	25	0	1
1)Expected number		12	18	6	8
1) Chi square		2.1	2.72	6	4.16
1) % of expected		142%	139%	0	17%
2) Number counted		35	47	1	3
2)Expected number		24	34	12	16
2) Chi square		5	5	10.1	11.6
2) % of expected		150%	138%	8%	19%
3) Number counted		33	35	1	1
3)Expected number		22	24	11	12
3) Chi square		5.5	5	9	10.1
3) % of expected		150%	146%	9%	8%
Total # expected		**58**	**76**	**29**	**36**
Total # counted		**85**	**107**	**2**	**5**
Average chi square		**4.2**	**4.24**	**8**	**8.6**
Average % of expect		**147%**	**141%**	**6%**	**15.00%**
SD of % expected		**4.62**	**4.36**	**4.93**	**5.83**
			P-value	**6.04468E-13**	

#33 ime4[sk2]/tm6c

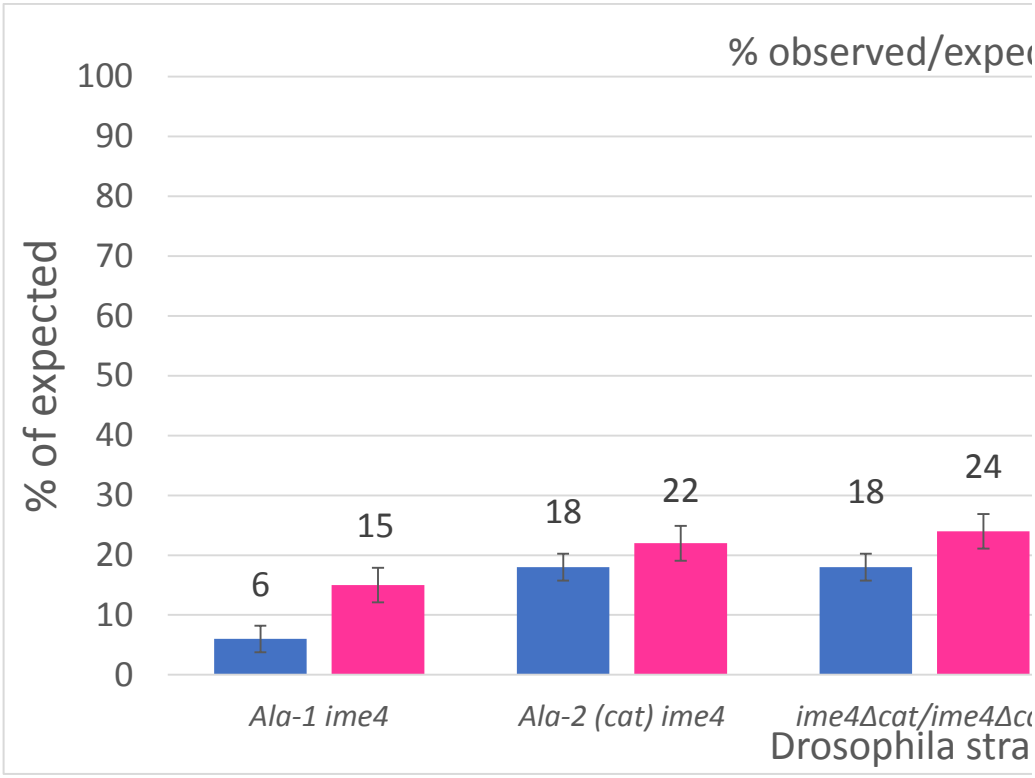
#) Drosoph

#88ime4 delta cat/tm6c

#89ime4 delta null/tm6c

*Calculated with 1 degree of freedom (3.84)

Expected = number of hatched embryos counted on grape-agar disc prior to transfer


% observed/expe
100
90
80
70
60
50
40
30
20
10
0
% of expected
6
15
18
22
18
24
Ala-1 ime4
Ala-2 (cat) ime4
ime4Δcat/ime4Δc
Drosophila stra

Strain	Line 4-2	Ala2-IME4/tm3		homozygotes	
Drosophila gender		male	female	male	female
1) Number counted		15	18	1	2
1)Expected number		10	14	5	7
1) Chi square		2.5	1.1	3.2	3.6
1) % of expected		150%	129%	20%	29%
2) Number counted		24	30	2	3
2)Expected number		18	22	9	11
2) Chi square		2	2.9	5.4	5.8
2) % of expected		133%	136%	22%	27%
3) Number counted		53	53	2	2
3)Expected number		36	36	18	18
3) Chi square		8	8	14.2	14.2
3) % of expected		147%	147%	11%	11%
Total # expected		**64**	**72**	**32**	**36**
Total # counted		**92**	**101**	**5**	**7**
Average chi square		**4.16**	**4**	**7.6**	**7.9**
Average % of expect		**143%**	**137%**	**18%**	**22.00%**
SD of % expected		**9.07**	**9.07**	**5.86**	**9.86**
		147	**147**	**20**	**29**
		150	**136**	**22**	**27**
		133	**129**	**11**	**11**
	SD Ala-2	9.073772	9.073772	5.859465277	9.865766
			P-value	**1.09965E-11**	

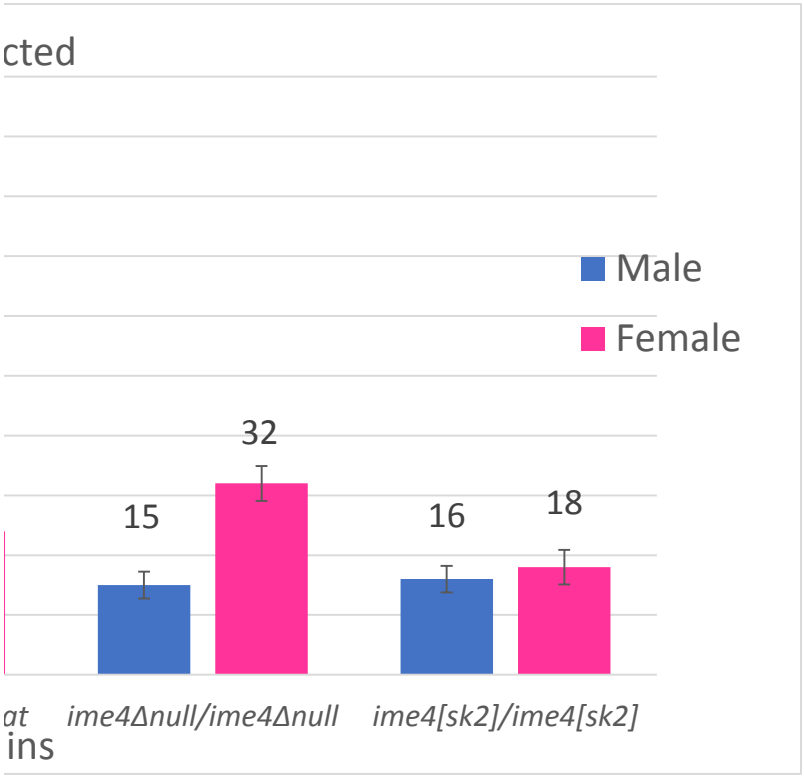
Strain	#33
Drosophila gender	
1) Number counted	
1)Expected number	
1) Chi square	
1) % of expected	
2) Number counted	
2)Expected number	
2) Chi square	
2) % of expected	
3) Number counted	
3)Expected number	
3) Chi square	
3) % of expected	
4) Number counted	
4)Expected number	
4) Chi square	
4) % of expected	
5) Number counted	
5)Expected number	
5) Chi square	
5) % of expected	
Total # expected	
Total # counted	
Average chi square	
Average % of expect	
SD of % expected	

ila counts accumulated over a period of 3 months and consolidated.

	Male	Female
Ala-1 ime4	6	15
Ala-2 (cat)	18	22
ime4Δcat/i	18	24
ime4Δnull/	15	32
ime4[sk2]/	16	18

SD #33

to food vial). Error bars indicate the standard error of the mean.


cted
Male
Female
32
15
16
18
at
ime4Δnull/ime4Δnull
ime4[sk2]/ime4[sk2]
ins

ime4[sk2]/tm6		homozygotes	
male	female	male	female
21	24	2	2
16	18	8	9
1.6	2	4.5	5.4
131%	133%	25%	22%
43	44	2	3
30	32	15	16
5.63	4.5	11.26	10.56
143%	138%	13%	19%
82	76	6	7
58	56	29	28
9.93	7.14	18.24	15.75
141%	136%	21%	25%
55	61	1	3
38	42	19	21
7.6	8.6	17.05	15.42
144%	145%	5%	14%
20	31	1	1
14	22	7	11
2.57	3.68	5.1	9.1
143%	141%	14%	9%
156	**170**	**78**	**85**
221	**236**	**11**	**15**
5.5	**5.2**	**11.23**	**11.25**
140%	**139%**	**16%**	**18.00%**
5.36	**4.61**	**7.73**	**6.38**
131	**133**	**25**	**22**
143	**138**	**13**	**19**
141	**136**	**21**	**25**
144	**145**	**5**	**14**
143	**141**	**14**	**9**
5.366563	4.615192	7.733046	6.379655163
		P-value	**7.12072E-27**

Strain	#88	ime4Δcat/tm6c	
Drosophila gender		male	female
1) Number counted		111	100
1)Expected number		82	76
1) Chi square		10.26	7.58
1) % of expected		135%	131%
2) Number counted		31	33
2)Expected number		22	24
2) Chi square		3.68	3.38
2) % of expected		141%	138%
3) Number counted		41	39
3)Expected number		28	28
3) Chi square		6	4.3
3) % of expected		146%	139%
4) Number counted		60	65
4)Expected number		44	50
4) Chi square		5.8	4.5
4) % of expected		136%	130%
5) Number counted		47	71
5)Expected number		32	48
5) Chi square		7	11
5) % of expected		147%	148%
6) Number counted		40	49
6)Expected number		28	36
6) Chi square		5.14	4.7
6) % of expected		143%	136%
Total # expected		**236**	**262**
Total # counted		**330**	**357**
Average chi square		**6.31**	**5.91**
Average % of expect		**141%**	**137%**
SD of % expected		**5**	**6.5**
		135	**131**
		141	**138**
		146	**139**
		136	**130**
		147	**148**
		143	**136**

homozygotes	
male	female
11	14
41	38
21.95	15.16
27%	37%
2	3
11	12
7.36	6.75
18%	25%
2	2
14	14
10.28	10.28
14%	14%
6	9
22	25
11.63	10.24
27%	36%
1	2
16	24
14.06	20.17
6%	8%
2	4
14	18
10.3	10.9
14%	22%
118	**131**
24	**36**
12.6	**12.25**
18%	**24.00%**
8.2	**11.6**
27	**37**
18	**25**
14	**14**
27	**36**
6	**8**
14	**22**
P-value	**3.98012E-33**

Strain	#89	ime4Δnull/tm6c		homozygo
Drosophila gender		male	female	male
1) Number counted		74	75	9
1)Expected number		56	58	28
1) Chi square		5.8	5	12.9
1) % of expected		132%	129%	32%
2) Number counted		9	9	0
2)Expected number		6	6	3
2) Chi square		1.5	1.5	3
2) % of expected		150%	150%	0%
3) Number counted		67	81	3
3)Expected number		46	56	23
3) Chi square		9.6	11.2	17.4
3) % of expected		146%	144%	13%
4) Number counted		25	17	2
4)Expected number		18	14	9
4) Chi square		2.72	0.64	5.4
4) % of expected		139%	121%	22%
5) Number counted		93	102	0
5)Expected number		62	72	31
5) Chi square		15.5	12.5	31
5) % of expected		150%	142%	0%
6) Number counted		38	20	3
6)Expected number		28	14	14
6) Chi square		3.6	2.6	8.64
6) % of expected		136%	143%	21%
Total # expected		**216**	**220**	**108**
Total # counted		**306**	**304**	**17**
Average chi square		**6.45**	**5.57**	**13.06**
Average % of expect		**142%**	**138%**	**15%**
SD of % expected		**7.6**	**10.9**	**12.86**
		132	**129**	**32**
		150	**150**	**0**
		146	**144**	**13**
		139	**121**	**22**
		150	**142**	**0**
		136	**143**	**21**
				P-value

tes
female
12
29
10
41%
1
3
1.33
33%
4
28
20.6
14%
4
7
1.3
57%
7
36
23.4
19%
2
7
3.6
29%
110
30
10.04
32.00%
15.54
41
33
14
57
19
29
3.54693E-31

Wild type flies

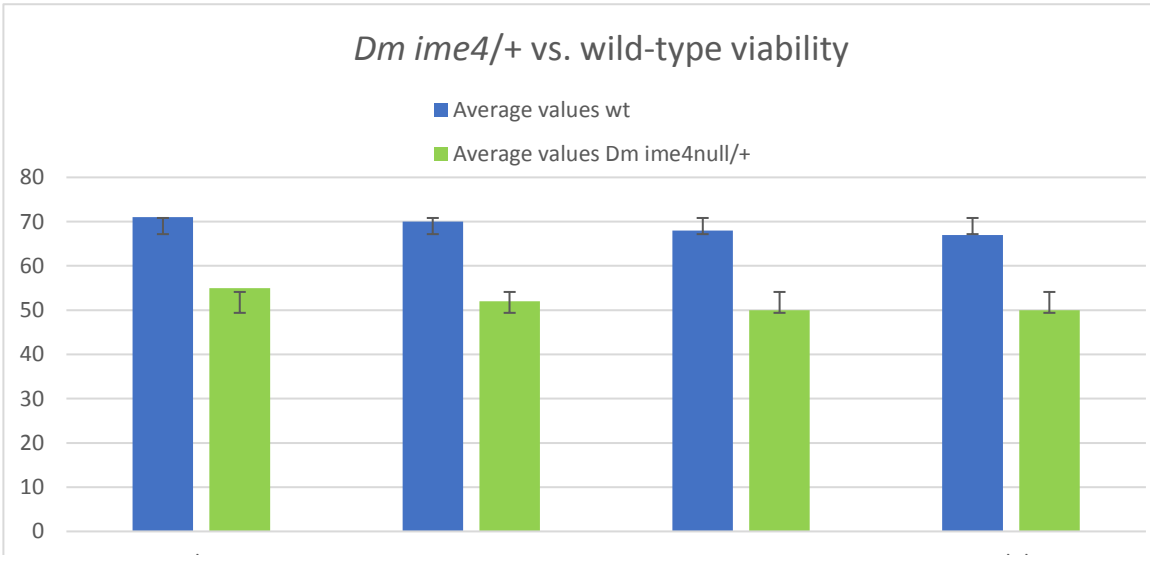
Agar down	Embryo	Larvae	Pupae	Adult	Adult/embryo
Biological r	81	81	81	77	95%
Biological r	65	61	60	60	92%
Biological r	70	70	67	67	96%
Biological r	75	75	69	68	91%
Biological r	64	64	61	61	95%
Average	71	70	68	67	94%
Standard D	7.106335	7.250287	8.414274	6.80441	0.01939072

Agar on sid	Embryo	Larvae	Pupae	Adult	Adult/embryo
Biological r	104	102	71	69	66%
Biological r	80	80	42	41	51%
Biological r	74	74	49	49	66%
	86	85	54	53	61%
Standard d	15.87451	12.03813	15.13275	14.42221	0.07071068

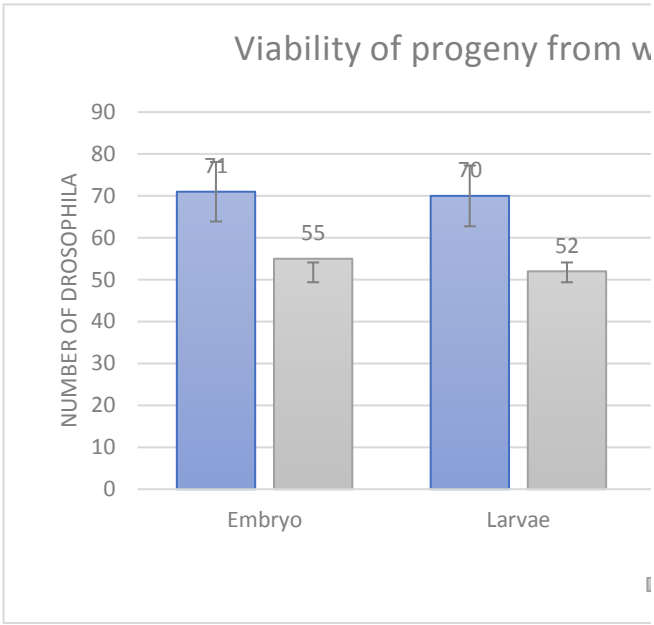
* Two females and one male were used in each cross (2.1 in manuscript).

Dm ime4 null /+ males crossed to virgin wild type females

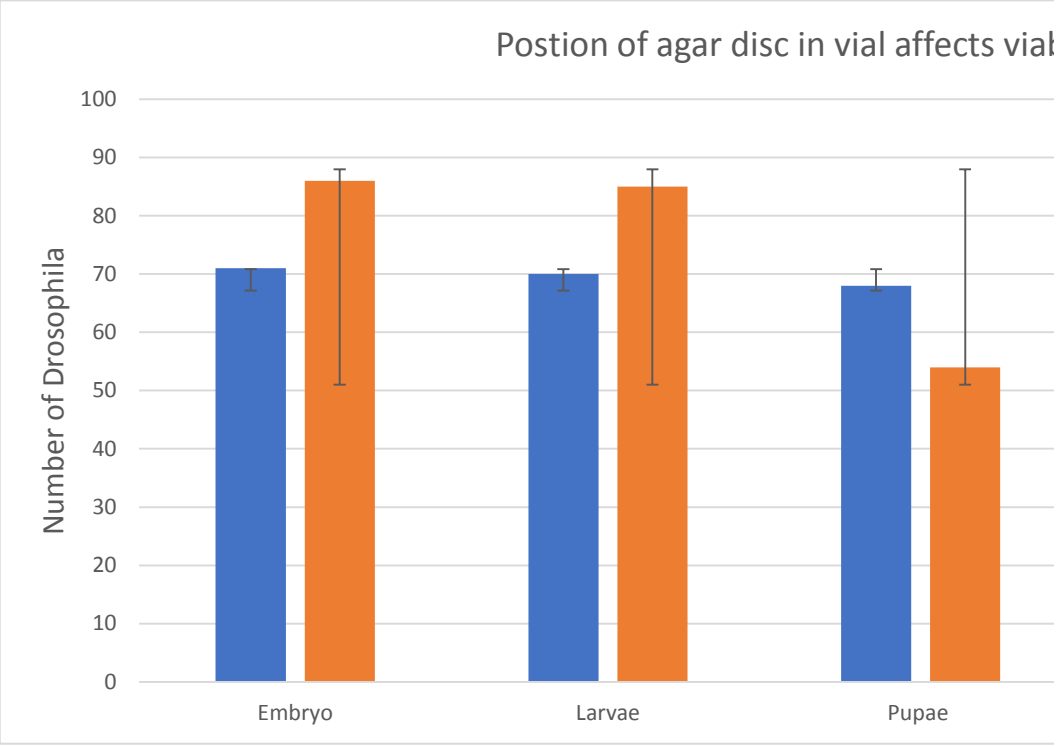
	Embryo	Larvae	Pupae	Adult	Adult/embryo
Biological r	56	56	52	52	93%
Biological r	49	46	46	45	92%
Biological r	59	53	53	53	90%
average	55	52	50	50	92%
std dev	5.131601	5.131601	3.785939	4.358899	0.01247219



Embryo	Larvae	Pupae	Adult

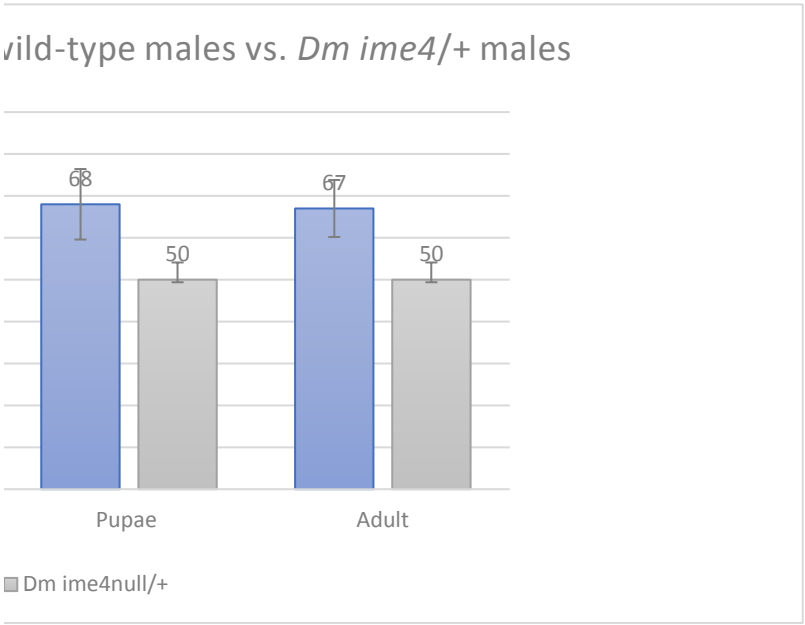


Average nu	Agar down	Agar on sid	Expected a	Expected agar on side	
Embryo	71	86	71	86	
Larvae	70	85	71	86	
Pupae	68	54	71	86	P-value
Adult	67	53	71	86	
X2	0.35	12.66			
P-value	1.5835E-05	P≥0.005	X2 cutoff =3.84		
adult/embı	94%	61%	12.66>>3.84, statistically significant difference		



Average values

	wt	*Dm ime4null* /+	Expected F	Expected side
Embryo	71	55	71	55
Larvae	70	52	71	55
Pupae	68	50	71	55
Adult	67	50	71	55
X2	0.35	0.45		
adult/embı	94%	92%		


ild-type males vs. Dm ime4/+ males
68
50
67
50
Pupae
Adult
Dm ime4null/+

Embryo-La	Larve-Pupa	Puape-adul	Embryo-adult
0.8726	0.00075	0.85537	0.000015

observed fl
70
68
67

e not due to chance alone.

observed s	expected fl	expected side
85	71	86
54	70	85
53	68	54