Dietary modulations to promote a regenerative response in the adult *Drosophila* limb

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SUMMARY
Some animals regenerate well, and some others do not. By working with animals that poorly regenerate, and investigate how to coax them to regenerate better, one can study factors that might have influenced the evolution of regeneration in some animals but not others. The adults of the fruit fly *Drosophila melanogaster* do not normally regenerate limbs. However, as larvae, they can regenerate the leg imaginal discs (Harris et al., 2020), raising the question whether this regenerative ability can be coaxed to re-emerge in adults. In this protocol, insulin, leucine, and glutamine are administered to promote regenerative response in the adult fly limb. Although the the regenerative response is not complete, the key finding is that it can be activated at all, and therefore enabling studies of the factors that contrain or promote regeneration in adult tissues. The limitation of the protocol is that inducing regeneration is difficult, and is likely to be influenced by multiple metbaolic and genetic factors that are not fully characterized yet. Therefore, careful technical considerations must be employed and implementation may involve several rounds of troubleshooting. For execution of this protocol, see Abrams et al., 2021.

BEFORE YOU BEGIN
This protocol assumes that the experimenter has prior experience working with fruit flies, and has a working fly setup in the lab.

Collect flies for the experiment
Timing: 2-5 days before experiment

1. Flies between 3-5 days old are used for this protocol.
2. On day 1, empty out fly stock bottles.
3. Over the next 5 days, collect newly eclosed flies and transfer them to a new bottle.

Note: We have not tested the effect of age. The considerations that went into the determining the age window used in this protocol are practical ones. Flies that are too young are harder to amputate because of the softer cuticles and the survival rate is correspondingly lower. Experimenting with flies that are too old means that we do not get to track them long enough.

With 3-5-day old flies, the survival rate is typically 100% after amputation, and 85% at 3 weeks after amputation.

KEY RESOURCE TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
L-Leucine	Sigma	L8912
L-Glutamine	Sigma	G3126
Insulin, human recombinant	Sigma	I0908
Deposited data		
Regeneration data - *Drosophila*	CaltechDATA	https://doi.org/10.22002/D1.2157
Experimental models: Organisms/strains		
OregonR wt *Drosophila* strains	Gift from Angela Stathopolous lab, Caltech Bloomington Drosophila stock center	RRID: BDSC_5
CantonS wt *Drosophila* strains	Gift from Kai Zinn lab, Caltech Bloomington Drosophila stock center	RRID: BDSC_64349
Software and algorithms		
Matlab codes to perform data analysis on the *Drosophila* measurements	eLife	https://cdn.elifesciences.org/articles/65092/elife-65092-fig5-code1-v3.zip

MATERIALS AND EQUIPMENT

Fly stocks

- Wild type strain CantonS (Bloomington Stocks #64349). We have also performed this protocol in OregonR (Bloomington Stock and #5, Abrams et al., 2021). **Note:** We have experimented with multiple CantonS strains and multiple fluorescent reporter lines. The regeneration induction can be observed in multiple genetic backgrounds, but the responsiveness to the treatment across fly strains and lines can vary, which makes sense given that metabolic state can be sensitive to genetic backgrounds. **Troubleshooting 1**

Equipment for surgery

- Small soft paint brushes for positioning flies (#1 or #3)
- Student Vannas Spring Scissors for amputation (Fine Science Tools, 91500-09)

- Dissection scope, equipped with a camera
- CO_2 anesthesia setup

Software for analysis

- ImageJ for image quantitation
- Excell spreadsheet for inputting measurements
- Matlab for data analysis

Solutions

- 10 mM L-leucine stock solution: dissolve 13.1 mg L-leucine in up to 10 mL final volume of ddH_2O. Store at 4ºC. Make fresh solution every 2 weeks.
- 10 mM L-glutamine stock solution: dissolve 14.6 mg L-glutamine in up to 10 mL final volume of ddH_2O. Store at 4ºC. Make fresh solution every 2 weeks.
- 0.1 mg/mL Insulin stock solution: dissolve 3 mg of insulin in up to 30 mL final vol of ddH_2O. **Note:** If insulin powders are hard to dissolve, adjust the pH to 3-4 with 5 mM HCl, and subsequently readjust pH to 7. Store at 4ºC. Make fresh solution every 1 week.

Leucine, glutamine, and insulin (LGI) master mix

Reagent	**Final concentration**	**Amount**
L-leucine stock solution (10 mM)	1.7 mM	1 mL
L-glutamine stock solution (10 mM)	1.7 mM	1 mL
Insulin stock solution (0.1 mg/mL)	33.3 µg/mL	2 mL
ddH_2O	n/a	2 mL
Total	**n/a**	**6 mL**

Store at 4ºC. Make fresh master mix right before use.

Fly food recipe part 1 Troubleshooting 2

Reagent	**Final concentration**	**Amount**
Agar	0.7%	136 g
Cornmeal	6.7%	1335.4 g
Active dry yeast	2.7%	540 g
Sucrose	1.6%	320 g
Molasses	8.2%	1.64 L
Calcium chloride dihydrate	0.06%	12.5g

Water	n/a	15.5 L

Fly food recipe part 2

Sodium tartrate tetrahydrate	0.75%	150 g
Propionic acid	0.5%	91.5 mL
Tegosept (12% stock)	0.1%	153.5 mL
Water	n/a	2.3 L
Total part 1 and 2	**n/a**	**20 L**

Note: Boil part 1 of the recipe, let the mixture cool to ~80ºC. Add part 2 and while continually mixing. Tegosept stock solution is 120.2 g Tegosept in 1 L 95% ethanol.

Note: Several standard fly food recipes are used across fly labs. We have not tested the effect of different food formulations. Since the regeneration-promoting factors are nutrients, it is plausible that the starting food composition can have an effect.

Critical:
Ethanol is flammable and should be kept away from open flame and used inside a fume hood.
Propionic acid is flammable and corrosive, should always be handled with proper PPE, kept away from open flame, and used inside a fume hood.
Tegosept is irritant and should be handled with proper PPE.

STEP-BY-STEP METHOD DETAILS

Prepare treated food

Timing: 15 min, do this the night before or at least 1 hour before starting the experiment
Add L-leucine, L-glutamine, insulin (LGI) mix to fly food.

1. Prepare the LGI master mix (see Materials).
2. Make the treated fly food:
    a. Place regular food vials in microwave for 5-10 s to melt the top layer. **Note:** It is easier to do this in small batches (~5 vials at a time).
    b. Pipet 200 µL of the LGI master mix into the melted top layer. Swirl gently with the pipet tip to spread and mix into the melted food.
    c. Place vials at 4ºC for 15 min or simply 30 min at room temperature to re-solidify.
3. Make sure vials are at room temperature before use. **Note:** Wipe out moisture forming on the side of the vial.

Amputate fly

Timing: 1.5-2 hours for 20-30 flies.

Amputation is performed under a dissection microscope.

4. Anesthetize flies. **CRITICAL:** Regeneration response can be affected by stress, and CO_2 is a known stressor (Nicolas and Sillans, 1989; Badre et al., 2005). Therefore, the duration of CO_2 exposure needs to be minimized. Minimizing CO_2 is a typical strategy in protocols studying aging and metabolism (e.g., Piper and Partridge, 2016; Landis et al., 2020; Colinet and Renault, 2012). We recommend anesthetizing 1-2 flies at a time, and keeping the CO_2 exposure to <5 min (our typical time is 2-3 min). Minimize the CO_2 flow rate to just enough from preventing the fly from walking (we typically use 2-3 liters/min). Prolonged anesthesia reduces responsiveness to treatment and lowers the survival rate. We recommend practicing the amputation until the user can comfortably amputate well under 5 min. **Troubleshooting 3**
5. Amputate a limb (Movie 1). Hold the fly steady using a soft brush, and quickly amputate the target limb with the other hand using spring scissors. Sometime the fly curls up when anesthetized. Just gently pry open the target limb using the soft brush or forceps. **Note:** We usually perform amputation across the tibia segment (red line in Figure 1a) because it is easier to access than the femur. See Figure 1c for a freshly amputated tibia. We have observed regenerative response from femur amputation, but never from tarsal amputation. We have not tried amputating the entire limb. **CRITICAL:** Different amputation methods can affect regenerative outcomes. Even in highly regenerating animal models, the way one inflicts injury influences the extent of regeneration (e.g., in axolotl, see Kragl and Tanaka, 2009; in zebrafish, see Dickover et al., 2013). In this potocol, we use surgical spring scissors (see Materials and Equipment).

Figure 1. Limb amputation. See Movie 1 that shows the amputation. In **B-C**, the scale bars denote 250 µm. **(A)** A drawing of a fly limb. We typically cut across the tibia segment (red line). **(B)** An uncut tibia. **(C)** A freshly amputated tibia.

Treatment

Timing: 15 min weekly for changing vials, 30-60 min for examining 20-30 flies.

1. For treatment: Place the amputated fly in a vial with treated food. For control: place the amputated fly in a vial with regular food. **CRITICAL:** We observe that amputated flies are more likely to get stuck in the food and die. To avoid this, the vials are stored horizontally at all times so the flies can rest on a non-food surface.
2. Keep <5 flies per vial. **CRITICAL:** Crowding is a stressor, and can reduce regenerative outcomes.

3. Treatment is performed for 5 d. At 5 days post-amputation, all flies (control and treated) were moved to fresh, regular vials. Subsequently, flies were moved to fresh vials every 5-6 days. Vials need to be refreshed regularly since the amputated flies resume mating and egg laying.
4. We tracked the flies for 3-4 weeks, and examined them at 3-5 days post amputation for wound healing phenotypes (Figure 2a) and tibia growth at 3-4 weeks after amputation (Figure 2b).

Single-fly tracking
Timing: 2-3 hours for 20-30 flies.

The bulk experiment can only capture the most dramatic, but rare, phenotype of tibia fully regrowing (Figure 2b). Tracking individual flies helps capture the entire extent of the phenotype. In Abrams et al., 2021, we find that almost half of the treated flies show growth of various extent (Figure 3). To track individual flies, we amputate the flies on different limbs (fore, mid, or hindlimb), and then house <5 flies per vial so we can trak each fly by which limb amputated and the sex (for instance, male forelimb versus female hindlimb). We do not find, so far, differences in the likelihood of regenerative response in fore, mid, or hindlimb, or between male and female.

5. Image the freshly amputated limb (the t0 data point). To image, position the amputated limb to a planar position by gently moving the fly using soft brush. **CRITICAL:** Use higher magnification and ensure the entire residual limb segment is in focus. We typically image the residual limb segment at 8X magnification. **Troubleshooting 4** The flies can still jitter even under anesthesia. To solve this problem, take a 15-30 second movie, and choose the in-focus frames to analyze. **Troubleshooting 5**
6. Label the image using the fly ID, created using the sex and limb amputated, e.g., female hindlimb, male forelimb. For simplicity, we use abbreviations: FF, FM, FH, MF, MM, MF (FF for female forelimb, MM for male midlimb, and so on).
7. Image each fly every 10-14 days. **CRITICAL:** The imaging time points balance between getting 2-3 times points for each fly, but not anesthetizing each fly too frequently since too much CO_2 exposure can lower survival and regenerative response.
8. We tracked all flies for 3-4 weeks or end of life, whichever occurs first.
9. See Quantification and Statistical Analysis for how to process the time-lapse imaging data.

EXPECTED OUTCOMES

The earliest sign that the treatment is working is whether or not the residual limb segment shows a melanized scab. Within 3 days post amputation, almost all if not all control flies develop a melanized scab (Figure 2A), which is a known wound healing response in *Drosophila* (Galkow and Krasnow, 2004). By contrast, 20-60% of treated flies (varies batch to batch) heal without a scab (Figure 2b). This modified wound healing response is not trivial, since scab formation is linked to immune response (Galkow and Krasnow, 2004). By 3-4 weeks after amputation, we would see, at 1% frequency, fully regrown tibias (Figure 2c). Fully regrown tibia is dramatic,

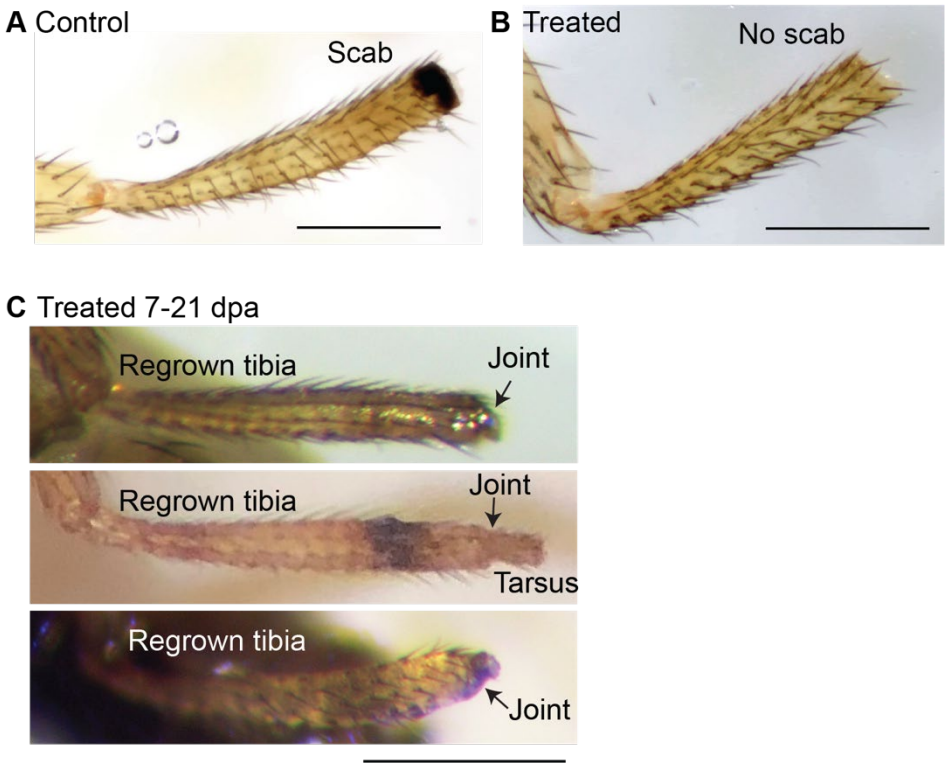

Figure 2. Expected outcomes. In these experiments, the fly limb was amputated across the tibia segment. All scale bars denote 250 μm. **(A-B)** Representative control **(A)** and treated **(B)** tibia stumps flies at 3 days post amputation. **(C)** Very rarely (at ~1%), at 1-3 weeks after amputation, we observed treated flies that fully regrow the amputated tibias. Figure 2C is reproduced from Abrams et al., 2021, licensed under CC BY 4.0

but occurs rarely, and can be sensitive to genetic backgrounds (we have observed this in CantonS and OregonR, but not in transgenic lines so far). The more robust assesment of the treatment effect, in addition to the modified wound healing (Figure 2a-b), is through quantitative single-fly tracking (Figure 3), which reveals that 30-50% of the treated flies show tibia regrowth of various extents. Current work in the lab is assessing tissues in the residual limb for another robust readout of regenerative response and assessing factors that can further enhance regenerative response.

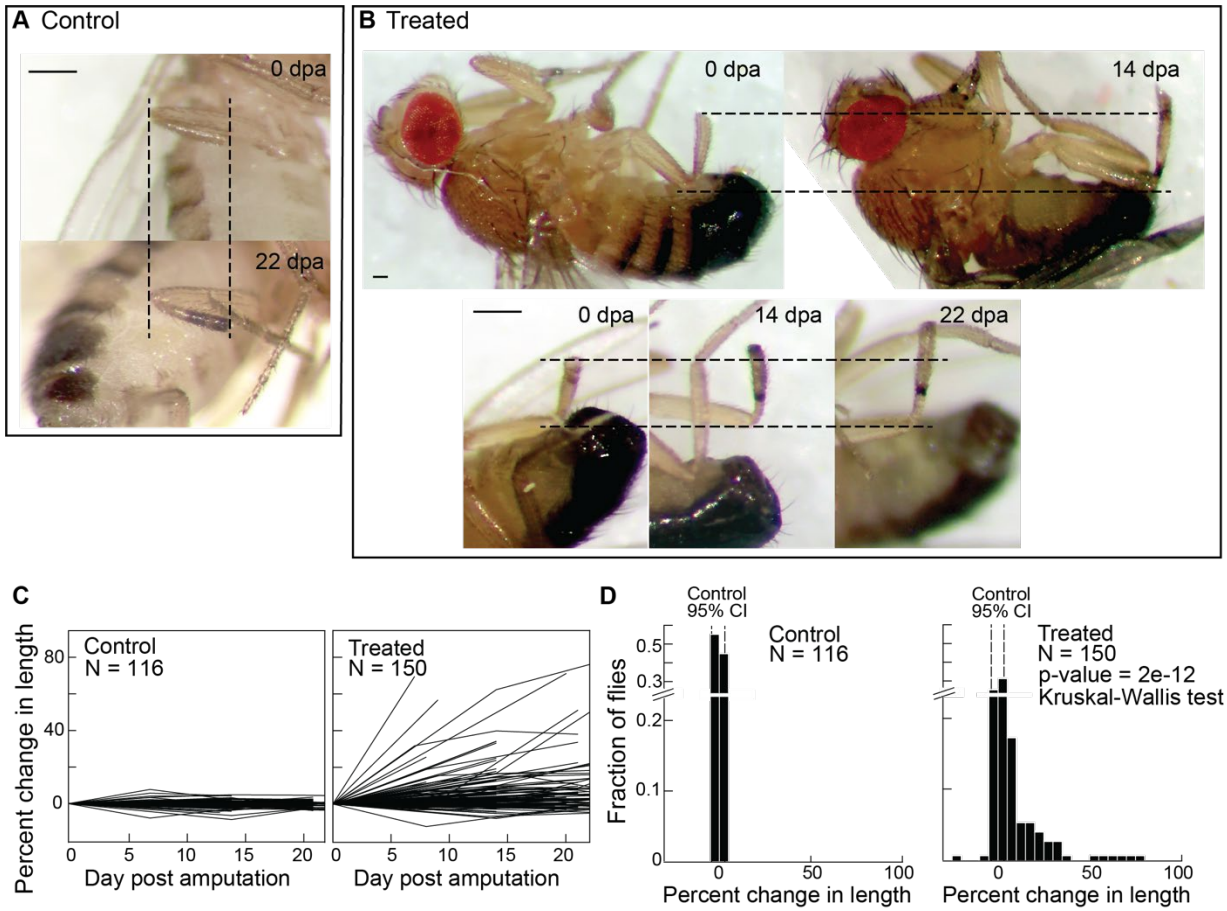

Figure 3. Expected outcomes from single-fly tracking

In these experiments, each fly was tracked and imaged every 10-14 days. **(A-B)** Representative time-lapse images of control tibia stumps **(A)** and treated **(B)** tibia stumps that show regrowth. **(C)** Change in tibia stump length over time. (**D**) Distribution of change in tibia stump length in control and treated flies. Percent change in length is the difference between the length at the final time point and 0 dpa, relative to the length at 0 dpa. CI: confidence interval. Statistical difference between control and treated distributions was evaluated using the nonparametric Kruskal-Wallis test. The p-value tests the null hypothesis that the data are drawn from the same distribution. Figure 3 are reproduced from Abrams et al., 2021, licensed under CC BY 4.0

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantify change in the length of limb stump from the time-lapse images

The goal of the quantitation is to measure the change in the length of tibia stump over the course of the experiment (Figure 3c-d). The movie frames that provide the planar view of the limb stump were selected for analysis. Length of tibia stump was quantified in ImageJ (Figure 4). For each fly, the change in the length of the tibia stump is computed as follows:

$$\%\ \text{change in length} = \frac{\text{length at time t} - \text{length at time 0}}{\text{length at time 0}} \times 100$$

The change in length is normalized within each fly to factor out variation in the initial stump length. Time 0 is the time immediately after amputation. Time t is a time from the subsequent time points.

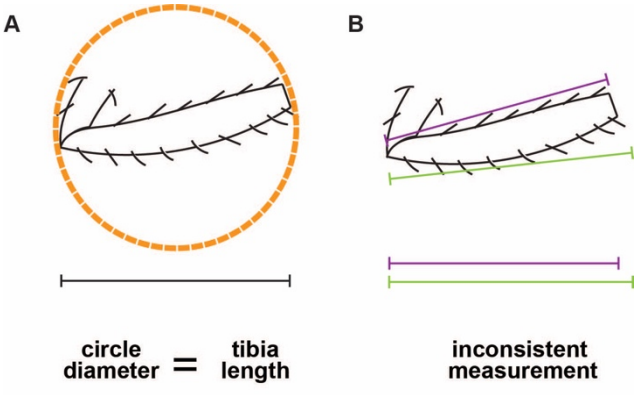

Figure 4. Quantification in *Drosophila* protocol. (A) To measure the length of the limb stump form the images, draw the smallest that can enclose the stump. The diameter of the circle is the length of the stump. **(B)** Measuring length as the diameter of the minimum enclosing circle of the limb stump is more robust to operator errors than measuring length by drawing a line, which is sensitive to the angles at which the line is drawn.

Statistical analysis

To assess whether the difference in the control vs treated distributions of change in the length of limb stumps (Figure 3d-e) are statistically significant, we performed a non-parametric Kruskal-Wallis test, which compares the samples from two or more groups according to their integer ranks.

TROUBLESHOOTING

Problem 1:

All treated limb stumps develop a melanized scab, or show a low percentage of scabless stumps.

Potential solution:

This suggests the treatment has not been performed correctly:

- Check that the stock solutions are not old. Check the concentrations, make sure insulin is dissolved, make sure it's been properly stored in the refrigerator. Try making fresh stock solutions.
- Check the concentractions in the Master Mix solution.
- Check the procedure of making the treated vials. Ensure the treatment master mix is well mixed. Ensure the master mix pipetted to the vials gets spread out over the top layer.

Alternatively, ensure amputation procedure and handling (e.g., amount of CO_2 used, duration of anesthesia) is as gentle as possible.

Problem 2:

Some control flies do not develop a melanized scab.

Potential solution:

In our experience so far, although the wt strains we have tested so far respond to injury as expected by developing a melanized scab, some flies carrying genetic markers or mutations can

show a baseline, low frequency of scabless healing. Regenerative response is a genetically encoded process, therefore it makes sense that genetic backgrounds can influence the propensity for mounting regenerative response.

Problem 3:
Treated flies show scabless healing, but do not regrow.
Potential solution:
- For the majority of flies, the regrowth is not dramatic, and can be difficult to spot by eye (Figure 3). We recommend performing quantiative single-fly tracking to fully determine extent of regenerative response.
- In our experience, reducing potential sources of stress improves the extent of regenerative response. We did this by minimizing the frequency, duration, and amount of CO_2 exposure, minimizing handling, and reducing housing density (<5 flies per vial).
- Fly lines. We observe variations in responsiveness to treatment across genetic lines and even across strains of the same genetic line (*e.g.*, CantonS strains from different labs).
- Fly food. Fly food recipes vary from lab to lab, and we have not tested how large this effect can be on the outcomes of the protocol. Since sugar and amino acids are essentially the treatment factors, it is plausible that the baseline protein and carbohydrate contents in the lab food can affect regenerative outcomes.

Problem 4:
Flies jitter and affect imaging quality and measurements.
Potential solution:
- Take a movie instead of snapshot. We solve the jitter problem by taking a 15-30 s movie for each limb stump, and chose the in-focus frames for analysis.

Problem 5:
Measurements of control are too noisy.
Potential solution:
- To determine the technical limits of the measurement protocol, we performed the entire protocol first on control, untreated flies. After rounds of optimization to reduce sources of errors, our control measurements yields a distribution with a near-zero mean (-0.3%) and 95% confidence intervals (CIs) of [–3.8, 3.2%]. This ~3.5% spread is the limit of the measurement protocol in our hand: any length change in the treated tibia stump that falls within the control CIs cannot be statistically distinguished as due to a real regenerative growth or simply measurement noise.
- We recommend that the experimenter first practices the protocol on control flies, and aiming for 5% or less of confidence intervals. If the control distribution is too large, ideas of troubleshooting include:
    - For each time point, measure several frames from the movie or frames from several replicate movies, and use the average.

- Make sure the limb stump is planar in the image quantified. The limb stump is planar if most if not all of the stump segment is in focus (looking at the sharpness of the bristles helps). If the limb stump is not planar in the image, do not use it. Take another movie.
- Ensure the way a stump is defined is consistent within the time-lapse images compared. Since each measurement is normalized within each fly, the segment definition only needs to be consistent within each fly. Therefore, use a morphological marker that is most obvious to you, which can be reproduced within the series of time-lapse images being compared. For instance, we use either the proximal or distal end of the femur/tibia joint to define the beginning of the stump segment.

LIMITATIONS OF THE PROTOCOL

How to promote regenerative responses is not fully understood. Therefore, relevant biological and technical parameters are not yet fully characterized that can affect whether or not regeneration can be successfully induced. Because of the nature of the phenomenon being studied, the experimenters may not be able to expect the experiments to work right away in their lab setups. It may need several rounds of troubleshootings. While we try to pen down every step here, there may be relevant factors that we take for granted in our lab setup that may critically vary across labs. On the flip side, working out these diffferences may be an opportunity for discovering new relevant parameters.

RESOURCE AVAILABILITY

Lead contact. Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lea Goentoro (goentoro@caltech.edu).

Materials, data and code availability. The datasets generated during the original study that developed these protocols and the associated codes for data analysis are available in Abrams et al., 2021. The associated raw data are available from the open repository CaltechDATA: https://doi.org/10.22002/D1.2157

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AUTHOR CONTRIBUTIONS

FHT conceived of and developed the fly treatment protocol. YL, AA, IL, LG further optimized the protocol, and developed the single-fly measurement protocol. LG supervised the work. All authors wrote and edited the manucripts.

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