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# Bipolar Head Regeneration Induced by Artificial Amputation in *Enchytraeus japonensis* (Annelida, Oligochaeta)

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**ABSTRACT** The Enchytraeida Oligochaeta *Enchytraeus japonensis* propagates asexually by spontaneous autotomy. Normally, each of the 5–10 fragments derived from a single worm regenerates a head anteriorly and a tail posteriorly. Occasionally, however, a head is formed posteriorly in addition to the normal anterior head, resulting in a bipolar worm. This phenomenon prompted us to conduct a series of experiments to clarify how the head and the tail are determined during regeneration in this species. The results showed that (1) bipolar head regeneration occurred only after artificial amputation, and not by spontaneous autotomy, (2) anesthesia before amputation raised the frequency of bipolar head regeneration, and (3) an extraordinarily high proportion of artificially amputated head fragments regenerated posterior heads. Close microscopic observation of body segments showed that each trunk segment has one specific autotomic position, while the head segments anterior to the VIIth segment do not. Only the most posterior segment VII in the head has an autotomic position. Examination just after amputation found that the artificial cutting plane did not correspond to the normal autotomic position in most cases. As time passed, however, the proportion of worms whose cutting planes corresponded to the autotomic position increased. It was suspected that the fragments autotomized after the artificial amputation (corrective autotomy). This post-amputation autotomy was probably inhibited by anesthesia. The rate at which amputated fragments did not autotomize corresponded roughly to the rate of bipolar regeneration. It was hypothesized then that the head regenerated posteriorly if a fragment was not amputated at the precise autotomic position from which it regenerated without succeeding in corrective autotomy.

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The Enchytraeida Oligochaeta *Enchytraeus japonensis* reproduces asexually only by spontaneous autotomy (fragmentation) in a mass culture condition. Among several hundred species of Enchytraeidae, only eight, including *E. japonensis*, have been reported to reproduce asexually by regeneration after fragmentation (Schmelt et al., 2000). When *E. japonensis* reaches 10–15 mm in length, the worm spontaneously autotomizes into 5–10 fragments, each of which is comprised of 5–10 segments. Each fragment regenerates a head (prostomium to segment VII) anteriorly and a tail posteriorly, in about 4 days, at 24°C (Myohara et al., '99). Regeneration of the present material is possible even from a fragment comprising only two trunk segments (unpublished). Moreover, it is possible to induce sexual reproduction by changing the culture condition. This enables research into embryonic development complementary to the study of regeneration (Myohara, 2004). Compared to hydras and planarians, which have been widely used in research into the regeneration of invertebrates, Annelida Oligochaeta is a more advanced triploblastic animal, as it has a well-developed ladder-like central nervous system, a closed blood-vascular system, a highly developed endocrine system, a coelom, and segmentation. Although many classical regeneration studies in Annelidae were conducted using Polychaetae and Oligochaetae (Christensen, '64; Herlant-Meewis, '64), for a long time thereafter, the field was barely advanced. Recently, though, some pioneering molecular biological studies have been reported on asexual regeneration in the aquatic Oligochaeta *Pristina leidyi*, which reproduces through paratomy (Bely and Wray, 2001), and on the early development of Annelidae (Seaver and Shankland, 2001; Seaver et al., 2001; Prud'homme et al., 2003). To date, however, the information is still fragmentary. To understand the formation and regulation of an animal's body plan, it is important to know whether or not the same mechanism determines the body axis in both embryogenesis and regeneration. How is an autotomized plane destined to form an anterior head vs. a posterior tail in *E. japonensis*? Although all spontaneously autotomic fragments regenerate a head anteriorly and a tail posteriorly without exception,

artificially amputated fragments occasionally regenerate a head posteriorly, resulting in bipolar head regeneration (Myohara et al., '99). The experiments in the present study are designed to clarify how the head is determined during the process of regeneration, by focusing on the bipolar head regeneration phenomenon. Our results suggest that the posterior head regenerates when regeneration occurs from a position other than the normal autotomy plane. Among regeneration studies of segmented animals, this is the first report to show that the position of the amputation within a segment is closely related to the type of structure formed. This phenomenon will serve as an interesting model for analyzing the axis determination in Annelidae.

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## MATERIALS AND METHODS

### *Animals and cultures*

Throughout the experiment, we used roughly 400–500 *E. japonensis*, an Enchytraeida worm cultured in our laboratory since 1995 as a closed colony. The cultures were kept at 24°C in 150-mm disposable Petri dishes (Falcon), each coated with 100 ml of 0.8% agar (Nacalai-Tesque) in Steinberg's solution designed for anuran larvae (58 mM NaCl, 0.67 mM KCl, 0.34 mM Ca(NO<sub>3</sub>)<sub>2</sub> 4H<sub>2</sub>O, 0.85 mM MgSO<sub>4</sub>7H<sub>2</sub>O, 4.6 mM Tris-HCl, pH 7.4) supplemented with 0.1% insoluble calcium carbonate salt. The worms had been fed powdered a rolled oats (Quaker Oats) every other day. The worms used for experimentation were placed in 60-mm disposable Petri dishes, each coated with 15 ml of 0.8% agar in Steinberg's solution.

### *Fragmentation*

#### **Decapitation**

To induce autotomy, the head was removed from a worm placed on a glass slide by cutting at intersegment VII/VIII with a disposable surgical blade (No. 19, Futaba). Because autotomy usually occurred within 24 hr after this operation (Inomata et al., 2000), the fragments were collected 24 hr after decapitation, and this collection time marked the beginning of day 0.

#### **Electrical stimulus**

Autotomy by electrical stimulus (Christensen, '64) was achieved by subjecting a worm to a direct current of 40 V (transformer Type RS-5A; Rikoh) for 0.5 sec several times on an agar plate dissolved with deionized water (Millipore). The fragments were transferred to experimental culture conditions within 10 min.

#### **Amputation**

The worms were amputated with a disposable surgical blade on a glass slide. The obtained fragments were placed on an agar plate or in deionized water. Some of the worms were anesthetized by a diluted L-menthol saturated solution (40%) for 10 min before amputation. The anesthetized worms transferred to agar plates or water awoke in 10–30 min.

### *Histology*

#### **Hematoxylin-eosin staining**

The worms and the fragments were fixed in Bouin's fluid for 4 hr, embedded in paraffin, and sliced into 5- $\mu$ m-thick sections. The sections were stained with hematoxylin and eosin using a routine method (Conn, '77).

## Whole-mount paracarmine staining

Worms were fixed in Bouin's fluid for 4 hr, washed several times in 70% ethanol, and stained with paracarmine (1 g carminic acid, 0.5 g aluminum chloride, 4 g potassium chloride, 70% ethanol to 100 ml) for 15 min. After washing in 70% ethanol followed by dehydration in a graded series of alcohol, the worms were mounted in a mixture of benzylalcohol and benzylbenzoate (1:2).

## Whole-mount $\alpha$ -bungarotoxinstaining

The worms and fragments were fixed in 10% formalin for 4 hr and were washed three times with phosphate-buffered saline (PBS; pH 7.4). The specimens were then preincubated for 6 hr in PBS containing 0.1% Triton X-100, 0.25% bovine serum albumin powder, and 0.05%  $\text{NaN}_3$  (Hessling and Westheide, '99). The specimens were washed five times in PBS, incubated with 5  $\mu\text{M}$  FITC-conjugated  $\alpha$ -bungarotoxin (Molecular Probes) for 1 hr, and washed in PBS three times.

## Scanning electron microscopy

The worms and the fragments were fixed for 4 hr in 5% glutaraldehyde in PBS containing 14% sucrose and then were post-fixed for 2 hr in 4%  $\text{OsO}_4$ . The specimens were dehydrated with alcohol, incubated in hexamethyldisilazane for 2 min, and air-dried at room temperature (Römbke and Schmidt, '99). The dried specimens were glued to a brass holder and gold-coated for observation by a JSM-5400LV scanning electron microscope (JEOL).

### *Statistical analysis*

Statistical analysis was carried out using contingency table analysis ( $\chi^2$  test for independence) first. If the  $\chi^2$  test results showed the results to be dependent on each other, Ryan's multiple comparison was used to compare further the two given groups in the data. The level of statistical significance in calculating Ryan's method was  $P=0.05$ .

## RESULTS

### *The morphology of bipolar head worms*

When *E. japonensis* sautotomizes spontaneously, each resultant fragment regenerates a head anteriorly and a tail posteriorly. However, when worm is amputated artificially, an extra head is occasionally formed posteriorly in place of a tail, resulting in a bipolar worm (Myohara et al., '99). On the other hand, a tail never forms anteriorly; without exception, a head is always regenerated at the anterior end of the fragment, regardless of any conditions. Our histological observation revealed that the posteriorly regenerated head had prostomium, mouth, brain, subesophageal ganglion, pharynx, and septal glands, like a normal head (Fig. 1A). The intestinal tract and the ventral nerve cord were formed continuously with the original part in the right D-V polarity (Fig. 1B). However, in most cases (90%, 63/70), the posterior head did not regenerate segment VII (data not shown). Also, in almost all cases, we found a short incomplete segment between the posteriorly regenerated head and the original part (Fig. 1D-F). The incompleteness of the intervening segments was determined by examining setae missing from these segments.

### *Induction of bipolar head regeneration by amputation*

To determine the cause of bipolar head regeneration, we examined the effects of three factors— anesthesia, method to obtain worm fragments (autotomy or amputation), and post-operative culture conditions. The obtained results are summarized in Table 1. Autotomy was induced either by removing the head (decapitation) or by applying an electrical stimulus (Inomata et al., 2000). We failed to obtain autotomy in any of the fragments that were anesthetized, even in fragments stimulated electrically. Among the fragments obtained by autotomy, those that did not contain a head or tail were selected out. The autotomized or amputated fragments were placed in either water or anesthetic solution for 1 hr and then were either transferred to an agar plate, or directly placed on agar. The autotomized fragments never regenerated a posterior head in any of the autotomy-inducing methods or under any of the culture conditions. Then, 10-mm-long worms, each consisting of about 60 segments, were amputated in the center of the body by a scalpel, either after or without 10 min of anesthesia. As shown in Table 1, the amputated fragments without anesthesia regenerated posterior heads at rates of 2.5% (cultured on agar) and 4.2% (placed in water) (amputation -/agar vs. -/water; statistically insignificant, Ryan's method). On the other hand, the amputated fragments with anesthesia regenerated posterior heads at rates as high as 24.0% (cultured on agar), and 34.6% (placed in water) (amputation +/agar vs. +/water; statistically insignificant, Ryan's method). Regardless of whether the worms were cultured on agar or in water, the frequencies of bipolar head regeneration were statistically different between unanesthetized and anesthetized fragments (amputation -/agar vs. +/agar, -/water vs. +/water; Ryan's method). Even when the culture period in water was prolonged to 2–8 hr, there were no significant changes in bipolar head

regeneration rates as compared with those cultured for 1 hr in water (data not shown).

Next, to examine the effect of amputation site in the body on posterior head formation, 10-mm-long worms were amputated artificially at different parts of the body (Fig. 2). The worms were anesthetized, amputated with a scalpel, cultured in water for 1 hr, and transferred to an agar plate (cf. Table 1; amputation, +/-water). As shown in Fig. 2, all fragments A, consisting of head segments (prostomium to V–VII), regenerated a posterior head. Only the result obtained in fragments A was statistically different from those in all other fragments (A vs. B, C, D, E; Ryan's method). Fragment B showed a lower incidence (14.8%) of posterior head regeneration than C (36.5%) and D (25.9%), and E showed the lowest posterior head regeneration (3.7%). Similarly, when worms 5 and 8 mm in length were amputated, fragments A regenerated a posterior head at the highest frequency; however, unlike the case with the 10-mm worms, no secondary peak was observed in fragments C or D (data not shown).

### ***The structures of head and trunk segments***

As described above, amputation at different segments of the body resulted in different rates of bipolar head regeneration. To analyze this phenomenon, we microscopically inspected the segment structures in various regions by scanning electron microscopy (SEM) and by whole-mount preparation of worms stained with FITC-conjugated  $\alpha$ -bungarotoxin. It was reported that autotomy always occurs at one specific position in each segment (Yoshida-Noro et al., 2000). SEM observation in the present study revealed that each trunk segment consists of four annuli and that autotomy occurs between the first and second annuli (Fig. 3A and B). Because bungarotoxin binds to nicotinic acetylcholine receptors, it is widely used as a tool for staining neuromuscular junctions in many animal species (Balice-Gordon and Lichtman, '90). The  $\alpha$ -bungarotoxin staining revealed that two broad bands and six narrow bands exist in each trunk segment, and that autotomy occurs just anterior to the second narrow band (Fig. 3C). TRITC-conjugated phalloidin staining showed that similar circular muscles are distributed equally in each segment. No special muscular structure is apparent in an autotomic position of the body wall (data not shown).

On the other hand, the structures of the head segments differed from those of the trunk region. In the head region, only segment VII consisted of the four annuli, as in the trunk segments (Fig. 3D). The numbers of bungarotoxin bands in the head segments were also lower than those in the trunk segments (Fig. 3F). When the worms were electrically stimulated, autotomy occurred only in segment VII among the head segments (Fig. 3E). The inset table in Fig. 3 clearly shows that only segment VII has structures similar to those of the trunk segments. The segments posterior to segment VII have essentially a uniform structure except for the several growing tail segments situated anterior to the pygidium.

### ***Amputation position and posterior head regeneration within a trunk segment***

Because bipolar head regeneration occurred only in artificially amputated worms, it seems plausible that an incorrect cutting plane caused posterior head regeneration. To test this possibility, artificially amputated fragments were compared with autotomized ones by exploiting the FITC-conjugated  $\alpha$ -bungarotoxin. Because the preliminary histological observation suggested that the cutting plane determines, within 8 hr after autotomy, whether a head or a tail will form, we examined whether the amputated fragments had correctively autotomized or not, at about 5 min after the amputation and again 8 hr later.

First, 10-mm-long worms were anesthetized and amputated at the center of the body. Then, the cut planes of the anterior fragments were examined (Fig. 4A). Approximately 5 min after the amputation, only 15% of the fragments had the correct autotomy plane. It was often observed that the remaining fragments, those amputated at the "incorrect" positions, autotomized in the segment next to the amputation plane, as if to remove the part of the segment that contained the cutting plane at a non-autotomic position (Fig. 4C and D). In this paper, we refer to this phenomenon as "corrective autotomy". When the worms were placed in water for 8 hr after amputation, the proportion of worms showing "corrective autotomy" decreased from 65% to 23.8% (gray bar, +/-0h vs. +/-8h water; statistically different by Ryan's method). In worms placed on an agar plate for the same period, the proportion decreased drastically, to 14.3% (gray bar, +/-0h vs. +/-8h agar; statistically different by Ryan's method).

Second, a new set of worms was amputated at the center of the body and without anesthesia (Fig. 4B). Approximately 5 min after the amputation, only 26.7% of the fragments had the correct autotomy plane, and 70% were in the process of "corrective autotomy". However, after 8 hr, the proportion of fragments having the correct autotomy plane had increased significantly, to 80%, even in water (white bar, -/0h vs. -/8h water; statistically different by Ryan's method), and the proportion of worms in water showing corrective autotomy decreased to 0% (gray bar, -/0h vs. -/8h water; statistically different by Ryan's method). Similarly, when worms were cultured on an agar plate, the proportion of those showing corrective autotomy decreased to 24.1% (gray bar, -/0h vs. -/8h agar; statistically different by Ryan's method).

When the fragments were cultured in water, the proportions of “autotomic position” (asterisk) and “corrective autotomy” (cross) 8 hr after amputation were statistically significantly different between anesthetized and unanesthetized worms (+/8h water vs. -/8h water; Ryan’s method). On the other hand, when the fragments were cultured on an agar plate, the difference was not significant (white bar and gray bar, +/8h agar vs. -/8h agar; Ryan’s method). Quite interestingly, the proportions of bipolar regeneration fragments (as shown by circles in Fig. 4) obtained by the same manipulation were roughly the same as those of the fragments having incorrect cutting planes at 8 hr after amputation, especially in anesthetized fragments. The anterior amputation plane of the posterior-half fragments simultaneously obtained in the same experiment also showed almost the same rate of corrective autotomy, but these posterior-half fragments never regenerated a tail anteriorly (data not shown).

### ***Regeneration from short head fragments***

As shown in Fig. 2A, short head fragments regenerated posterior heads at an extraordinarily high rate. We studied the relationship between the status of the amputation plane and posterior head regeneration. Only a few autotomized head fragments, consisting of the prostomium through segment VI (autotomized at the autotomic position in segment VII), were obtained (“VI/VII” in Fig. 5A) by the electrical stimulus. These and the autotomized fragments containing longer heads never regenerated a head posteriorly (“VI/VII” through “IX/X” in Fig. 5A; cf. Fig. 3D and E).

For comparison, the 5-mm-long worms were amputated at different head and trunk intersegments after anesthetization, and were cultured on an agar plate (Fig. 5B; cf. Table 1; amputation, +/agar). Almost none of the head fragments cut at the IV/V intersegment formed even a blastema, and no regeneration was observed. The head fragments cut at intersegment V/VI never regenerated a tail posteriorly. As a result, 100% of the worms had bipolar heads. The head fragments cut at intersegments VI/VII and VII/VIII regenerated 5-mm worms and cultured on an agar plate in order to examine whether or not the shortness of the fragments might determine posterior head regeneration. As a result, 92.9% of fragments regenerated a head anteriorly and a tail posteriorly (4 seg in Fig. 5B). Also, all four-segment fragments obtained by electric-current-induced autotomy regenerated normally (“4 seg” in Fig. 5A). Similarly, amputated four-segment tail fragments (97/97) regenerated an anterior head without exception (data not shown).

### ***Histological observation of the regenerating head fragments***

The shortest head fragments obtained by current-induced autotomy in segment VII were only slightly longer than the fragments artificially amputated at the intersegment VI/VII. However, the regeneration patterns between the autotomous and the artificially amputated fragments were completely different (% posterior head: 0% in “VI/VII” of Fig. 5A vs. 84% in VI/VII of Fig. 5B; statistically different by Ryan’s method). Because the bungarotoxin band pattern in the head segments is different from that in the trunk segments (Fig. 3), bungarotoxin staining could not be used to identify the exact position of the amputation in head segments. We therefore used SEM to compare the autotomized and amputated fragments. In autotomized fragments, the anterior plane was round and its center was open slightly at approximately 5 min after autotomy (Fig. 5C), while the posterior plane was more protrudent and its center was completely closed (Fig. 5D and E). On the other hand, in most of the fragments artificially amputated at intersegment V/VI or VI/VII, the posterior plane was not closed completely at approximately 5 min after amputation, and a part of the intestine surrounded by the chloragogen tissues was exposed (Fig. 5F and G). It should be mentioned that corrective autotomy was not observed in these head segments, in contrast to the trunk segments.

### ***Fate of the bipolar head worms***

The fate of the bipolar head worms was traced after posterior head regeneration. Although the bipolar head worms did not grow because they lacked the growth zone normally located in the tail region, the worms usually spontaneously autotomized at the original fragment region as early as 1–6 days after the initial amputation. The posterior-half fragments thus formed, including the posterior head, regenerated the head anteriorly from the anterior autotomy plane. This resulted again in a bipolar head worm. In turn, these bipolar head worms spontaneously autotomized again, and the posterior fragments regenerated the anterior head, resulting again in a bipolar head worm. After this third autotomy, all the posterior fragments died, probably because they were too small to heal the wound. During the period when posterior-half fragments regenerated a head anteriorly, all anterior-half fragments regenerated a tail posteriorly, resulting in normal worms.

So far, we have failed to induce anterior tail regeneration in the posterior fragment of bipolar head worms under any conditions, including anesthetization, artificial amputation, culture in water, and so on. On the other hand, the anterior-half fragments derived from bipolar worms occasionally regenerated the head posteriorly by artificial amputation, as in normal worms.

## DISCUSSION

### *Bipolar head regeneration*

In this paper, bipolar head regeneration is defined as the formation of a heteromorphic head at the posterior end. In hydras and planarians, many researchers have reported that bipolar head regeneration occurs in very short fragments made along the anterior–posterior axis and in chemically treated fragments (Berrill and Karp, '81). Posterior head regeneration was also reported to occur in the strobila larva of the Scyphozoa *Aureliaaurita* (Kroiher et al., 2000), the Polychaeta *Sabella melanostigma* (Fitzharris and Lesh, '69), and the aquatic Oligochaeta *Dero limosa* (Hyman, '16). In the terrestrial Oligochaeta *E. japonensis*, a head occasionally formed at the posterior end. Because anterior head regeneration is extremely stable in this species, no bipolar tail worm has so far been observed. It was reported that in another Oligochaeta, *Eisenia foetida*, the tail sometimes formed anteriorly but the head never formed posteriorly (Gates, '49, '50). Although the mechanism of head and tail determination has not been clarified, it seems that the different Oligochaeta species have different mechanisms.

### *Apparent absence of global control over head– tail formation*

It has been widely accepted that hydras and planarians have a head-forming gradient that is high in the head region and decreases toward the tail, because the quality and quantity of head regeneration decrease from the head toward the tail (Rose, '70). The result shown in Fig. 2 seems to indicate that a head-forming gradient may exist also in *E. japonensis*. However, this result was obtained only when corrective autotomy was effectively inhibited by anesthesia before the amputation (cf. Table 1; amputation, -/agar vs. amputation, +/water). Because spontaneous autotomy does not usually occur in the head region, the apparently existent gradient for bipolar head formation is considered to be an illusion reflecting the rate of corrective autotomy, which is extremely rare or totally lacking in the head region. Moreover, none of the newly formed posterior heads ever reversed the original AP axis of the worm, as shown by the regeneration after separation of bipolar worms, where the anterior head was formed in whichever fragment contained the posterior head. Quite similar results were observed in a Polychaeta (Fitzharris and Lesh, '69).

### *Manipulation causing bipolar head regeneration—inhibition of corrective autotomy*

The results shown in Table 1 suggest that artificial amputation is a prerequisite to induce bipolar head regeneration and that anesthesia before amputation will increase the frequency. When anesthetized worms were amputated in the trunk region and cultured in water, the corrective autotomy rate was quite low as compared with the rate in unanesthetized worms (Fig. 4A and B). Anesthetization also inhibited the rate of spontaneous autotomy induced by either decapitation or electrical stimulus (data not shown). It is highly probable that the reason why anesthesia inhibited corrective autotomy is that it temporarily paralyzes contraction of the circular muscles.

The rates of corrective autotomy on the agar plates did not statistically differ between fragments that were anesthetized and those that were not. Without anesthesia, the increase in “autotomic position” in the water culture was as high as that observed in the agar culture. This might show that unanesthetized fragments could respond even when placed under water. Menthol, which was used as the anesthetic in the present study, generates a sense of coldness in animals by activating the transient receptor potential family of ion channels (Peier et al., 2002), causing muscle paralysis in some animals. Although the possibility cannot totally be excluded from the present study, it is not likely that the menthol disrupted positional information directly, because the autotomized fragments that were treated with anesthesia after autotomy never regenerated a posterior head (Table 1).

### *Regeneration of head fragments*

The amputated head fragments regenerated a head posteriorly at an extremely high rate (Figs. 2 and 5B). Additionally, we discovered for the first time that the structures of segments I (= peristomium) to VI differed from those of the more posterior segments; in particular, the former head segments had no autotomic position (Figs. 3 and 5). Similarly, in the paratomic Oligochaeta *D. limosa*, none of the four head segments has an autotomic position, while each trunk segment does (Reyners and Reyners, '73). It is unlikely that the high rate of bipolar head regeneration in the amputated fragments at intersegments V/VI and VI/VII is attributable to the fragments having too few segments, since the amputated four-segment trunk fragments regenerated normally (Fig. 5B). Thus, we have concluded that the cause of bipolar head regeneration in head fragments is that the head segments are incapable of corrective autotomy and differ from the trunk segment in structure.

### *Posterior head is regenerated from a position other than that of autotomy*

The overall results suggest that a posterior head is formed when regeneration starts at a nonautotomic position.

The incomplete short segments observed at the base of the posterior head in bipolar head worms might be vestiges of the segments that were produced by the amputation at the non-autotomic position (Fig. 1D–F and Fig. 6). We conducted an amputation experiment at various positions within one trunk segment, but the results did not differ according to the cutting positions (data not shown).

Strangely, the anterior amputation plane always regenerated a normal head, no matter where the amputation occurred within a segment and regardless of whether anesthesia was used or not. These results strongly suggest that a head always forms from any anterior or posterior amputation plane (correct or incorrect), except when the amputation is in the posterior plane and its plane is correct, in which case a tail forms.

### ***The determination of head or tail***

Our current model is depicted in Fig. 6, where head or tail formation is determined locally by the nature of the exposed plane. Although the mechanism for head vs. tail determination remains unresolved in the present study, it is probable that the arrangement of position-specific tissue in one segment may be involved.

The undifferentiated stem-like cells (neoblasts) are attached to the ventral–posterior septa in every trunk segment, but they are not found at all in head segments. In correctly autotomized fragments, the neoblasts are located near the posterior plane. This phenomenon prompted us to consider whether or not neoblasts could direct the formation of a tail blastema. However, it must be recalled that the head fragments that lacked neoblasts and that autotomized at segment VII regenerated the tail posteriorly (“VI/VII” in Fig. 5A). This result suggests that neoblasts are not the sole mechanism to directly determine the tail blastema. Old experiments with Oligochaetae reported that a transplanted nerve cord at the lateral or anterior dorsal amputated plane induced ectopic head regeneration (Herlant-Meewis, '64). Thus, another possible cause of posterior head regeneration is involvement of a nerve cord. A fine-tuned histological observation and molecular biological analysis, both currently under way, might well clarify the cause of head and tail determination at specific positions within a segment.

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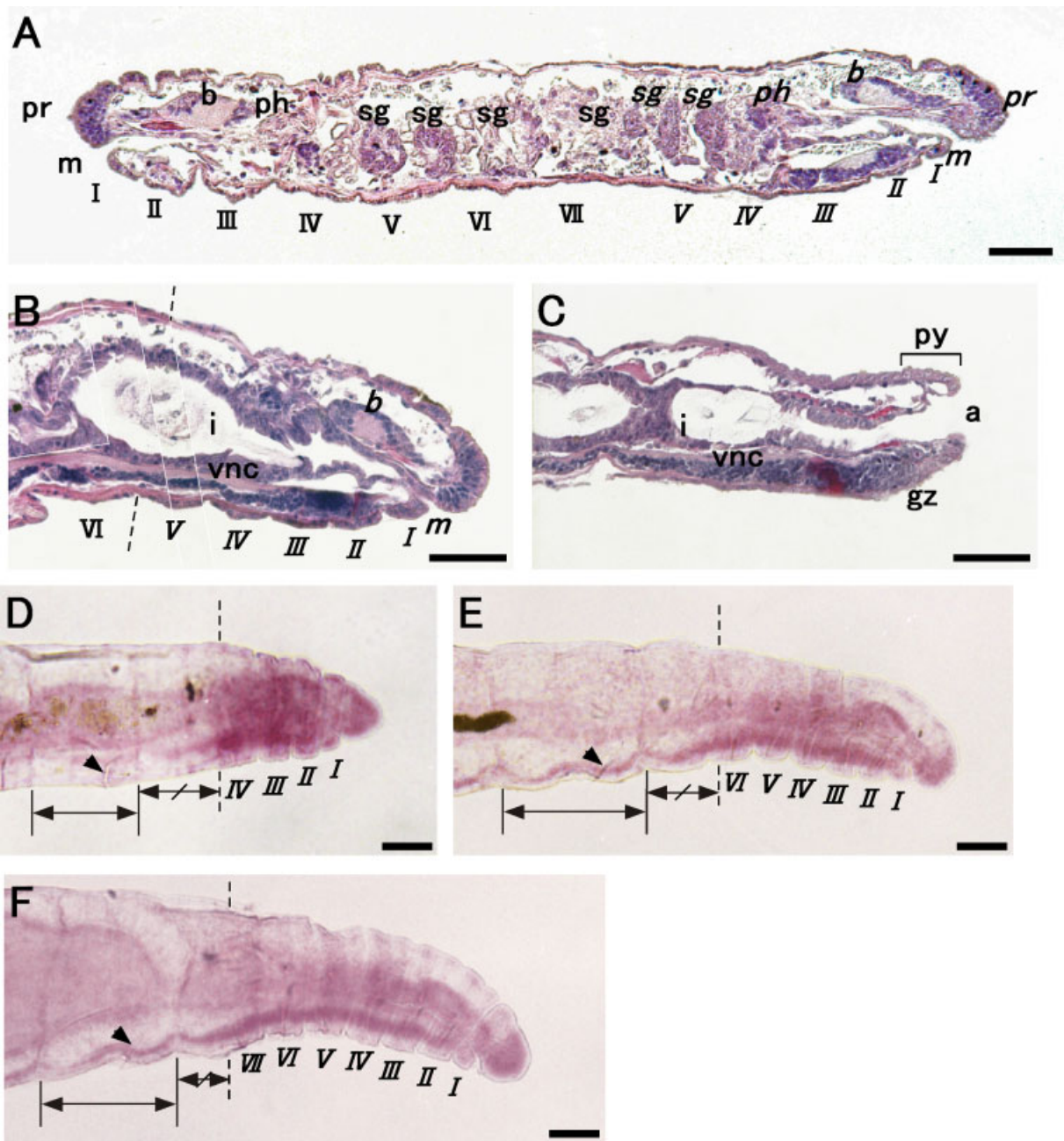


Fig.1. Histological observation of bipolar head worms. Sagittally sectioned (A–C) and paracarmine-stained (D–F) whole-mount preparations of *Enchytraeus japonensis*. In all figures, anterior is towards the left and the scale bar is 50  $\mu$ m. (A) Bipolar head worm consisting of the original head (I–VII) and a posterior head (I–V). (Regenerated parts are indicated in italics.) pr, prostomium; b, brain; ph, pharynx; sg, septal gland; m, mouth. (B) Posterior head (I–V) regenerated from head fragment (VI). Dotted line indicates the border between the original fragment (left) and the regenerated part (right). The intestine (i) is connected continuously to the posterior mouth (m). Also, the ventral nerve cord (vnc) is continuous between the stub and the regenerate. Since the worm has no anus, the excrement cannot be discarded and accumulates in the intestine. White lines indicate the boundaries of four photographs taken from serial sections. (C) Normal tail. The anus (a) opens at the end of pygidium (py). Many proliferating cells are observed in the growth zone (gz). (D–F) The “incomplete short segments” (slashed bipolar arrows) observed between the nearest neighboring normal complete segment (bipolar arrow) in the stub and the regenerated posterior head. The incompleteness of the segment was determined by the position or the absence of setae. Arrowheads show normal setae in the next segments. The length of each “incomplete short segment” varied from worm to worm. The worm in (D) regenerates a posterior head to segment IV, while those in (E) and (F) regenerate to VI and VII, respectively.

TABLE 1. Regeneration from the posterior cut end after autotomy or artificial amputation

Anesthesia/ culture	Autotomy (induced by)							
	Decapitation				Amputation			
	Electrical stimulus		Electrical stimulus		Electrical stimulus		Electrical stimulus	
	-/agar (N = 127)	-/agar (N = 108)	-/water (N = 150)	-/ane. sol. (N = 134)	-/agar (N = 119)	-/water (N = 118)	+ /agar (N = 121)	+ /water (N = 133)
Tail	99.2	100	100	100	90.8	93.2	57.9	55.6
Undetermined	0.8	0	0	0	6.7	2.5	18.2	9.8
Head	0	0	0	0	2.5	4.2	24.0*	34.6

Bipolar head regeneration was observed only by artificial amputation and not by autotomy. In amputated fragments made without anesthesia, posterior head regeneration occurred. But the rates of posterior head regeneration were always low regardless of the culture conditions (-/agar and -/water). The anesthesia given before amputation exceedingly raised the rate (+/agar and +/water). N: the number of operated worms. ane.sol.: autotomized fragments were cultured in the water containing anesthetizing menthol. Values are expressed in percentages. The significance probability by  $\chi^2$  analysis is less than 0.001.

Tail: normal tail is formed; Undetermined: unidentified structure or abnormal tail is formed; Head: the head is formed posteriorly.

\*Contained one worm which regenerated head and tail simultaneously at the cut end.

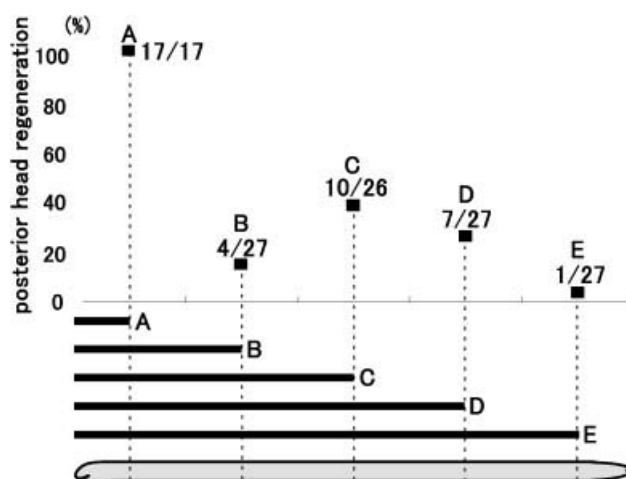


Fig. 2. The frequency of posterior head regeneration from different regions of the body. The schematic diagram at the bottom shows the amputated sites in a 10-mm worm (consisting of about 60 segments). Fragment (A) was obtained by amputating at 0.5 mm from the anterior end (consisting of about seven segments). Likewise, (B) was cut at 2.5 mm (ca. 20 segments), (C) at 5 mm (ca. 30 segments), (D) at 7.5 mm (ca. 40 segments), and (E) at 9.5 mm (ca. 50 segments) from the anterior end. The frequencies of bipolar head regeneration depended on body region ( $P < 0.001$ ,  $\chi^2$ ). All of the A fragments regenerated a posterior head, resulting in short bipolar worms. The number shows bipolar regeneration/operated worms.

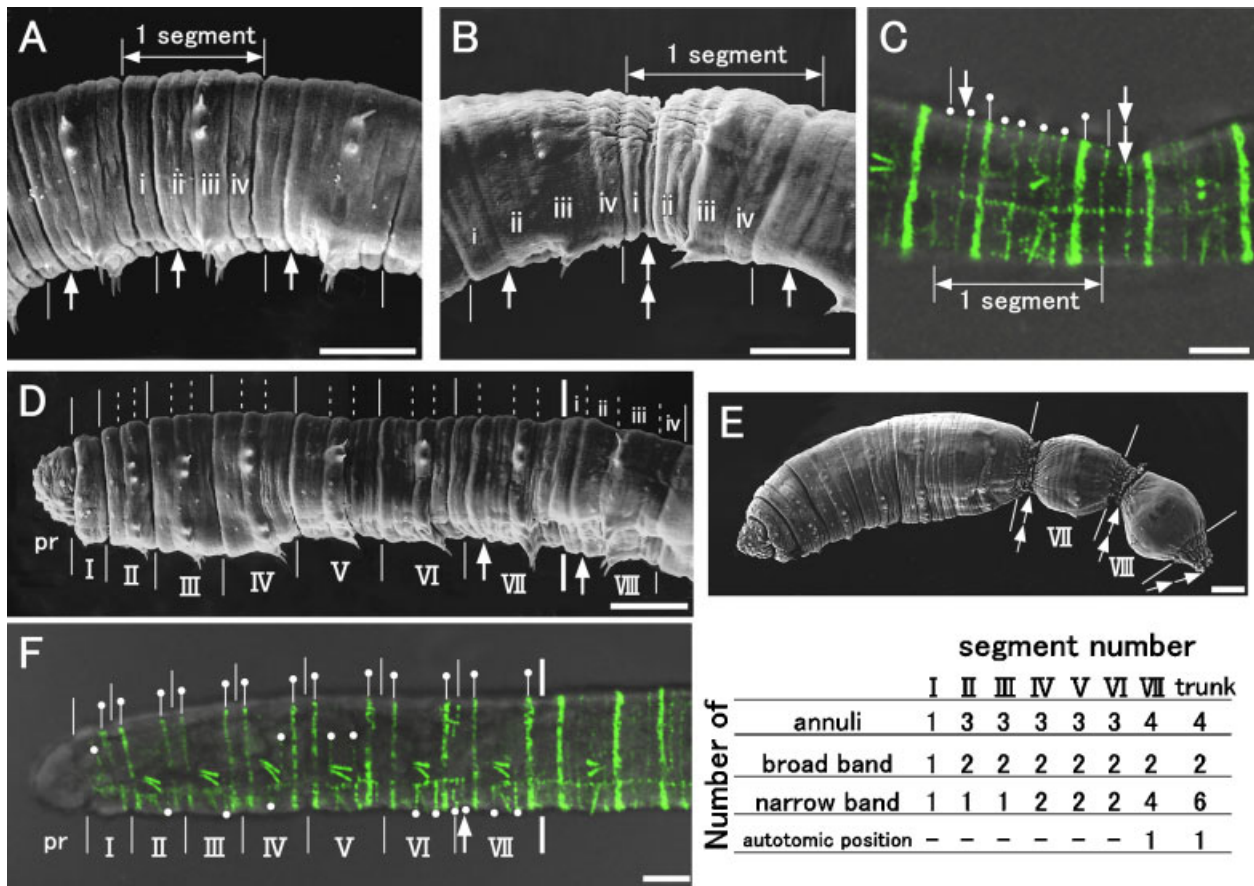


Fig. 3. Gross morphology of head and trunk segments. (A) SEM image of trunk segments. Each trunk segment consists of four annuli (indexed by lowercase Roman numerals) and has one autotomic position (arrow). Vertical bars indicate inter-segments. (B) SEM image of autotomizing trunk segment. The autotomy (double arrows) occurs between the first and the second annuli. (C) Autotomizing (double arrows) trunk segments stained by fluorescent bungarotoxin. Two broad bands (white closed circle with bar) and six narrow bands (white circle) are observed in one segment. (D) SEM image of head segments. The dotted lines indicate interannuli. Segment I (= peristomium) following prostomium (pr) consists of only one annulus. Each II–VI segment consists of three annuli. Only segment VII consists of four annuli in the head, as do the trunk segments. In this photograph, it is not easy to identify the most posterior annulus of segment II. (E) Autotomizing head fragments. The autotomic positions in both segments VII and VIII are constricted, and autotomy was completed in segment IX. This fragment consists of prostomium, peristomium (I), six head segments (II–VII), trunk segment VIII, and trunk segment IX's most anterior annulus. (F) Head segment stained with fluorescent bungarotoxin. Each II–VI head segment has two broad bands (white closed circle with bar) similar to the trunk segments. A summary of the segment structure is shown in the inset table.

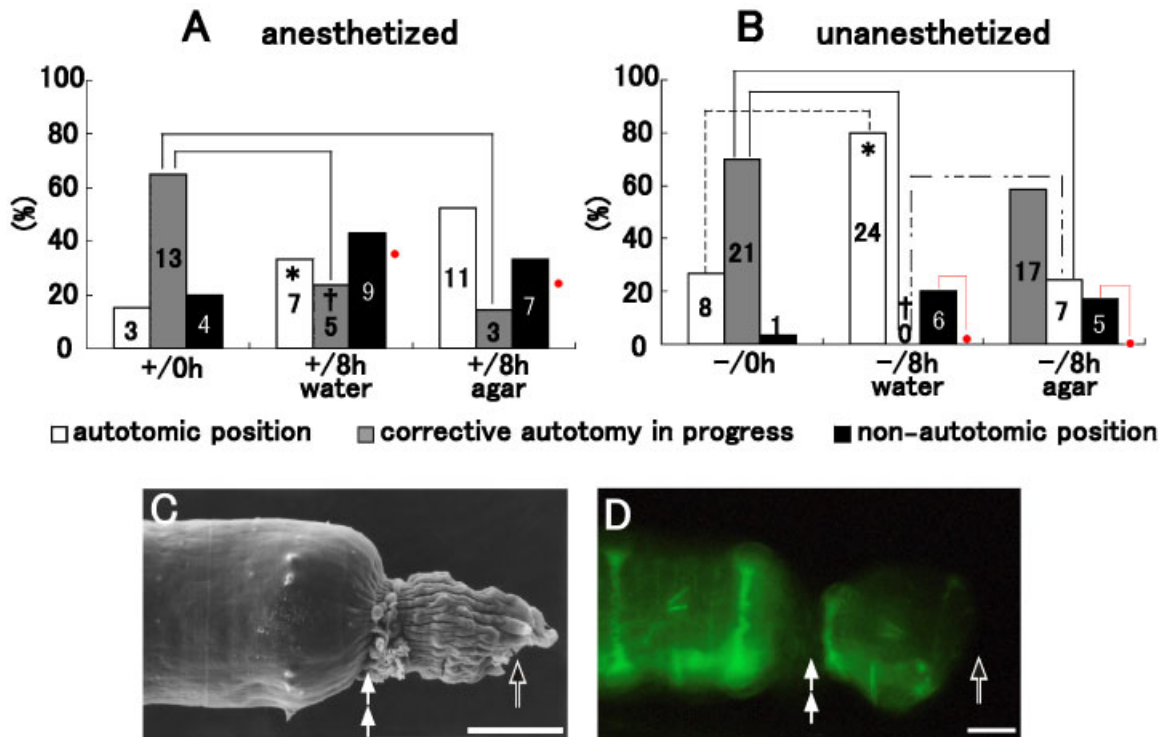


Fig. 4. Relationship between amputation position and bipolar head regeneration in trunk segments. Changes in cut position between operated worms approximately 5 min after amputation (0h) and 8 hr later. The frequencies of cut positions in a segment depended on the periods after amputation and on the culture conditions ( $P < 0.001$ ,  $\chi^2$ ). (A) Anesthesia was used (+) in this experiment. Five minutes after amputation, only 15% of the fragments were cut precisely at the autotomy plane (white bars), and the other fragments had an amputation plane at a non-autotomic position, either with or without the autotomizing contraction just anterior to the amputation plane (gray bars and black bars). Among worms cultured under water (+/8h water) or on agar plate (+/8h agar) for 8 hr after amputation, 33.3–52.4% had the correct autotomy plane. (B) On the other hand, if the worms were cut without anesthesia (-), the percentages of fragments having the correct autotomy plane significantly increased with time (26.7% at 5 min after amputation (-/0h) and 58.6–80% after 8 hr of underwater culture (-/8h water)). The circles next to the black bars indicate the rates of bipolar head regeneration assessed 5 days later in each group. Brackets indicate the statistical differences proven by Ryan's method between two comparative groups in each graph. Each comparison of significance between graphs A and B is expressed with the same symbols (asterisk and cross). SEM image (C) and bungsarotoxin staining (D) of a fragment amputated without anesthesia showed that the worms were autotomizing at the correct autotomic positions (double white arrows). Black arrows indicate the original amputated positions. Note that the broad band does not exist at the posterior end near the black arrow in (D), showing that the amputation was made in a nonautotomic position.

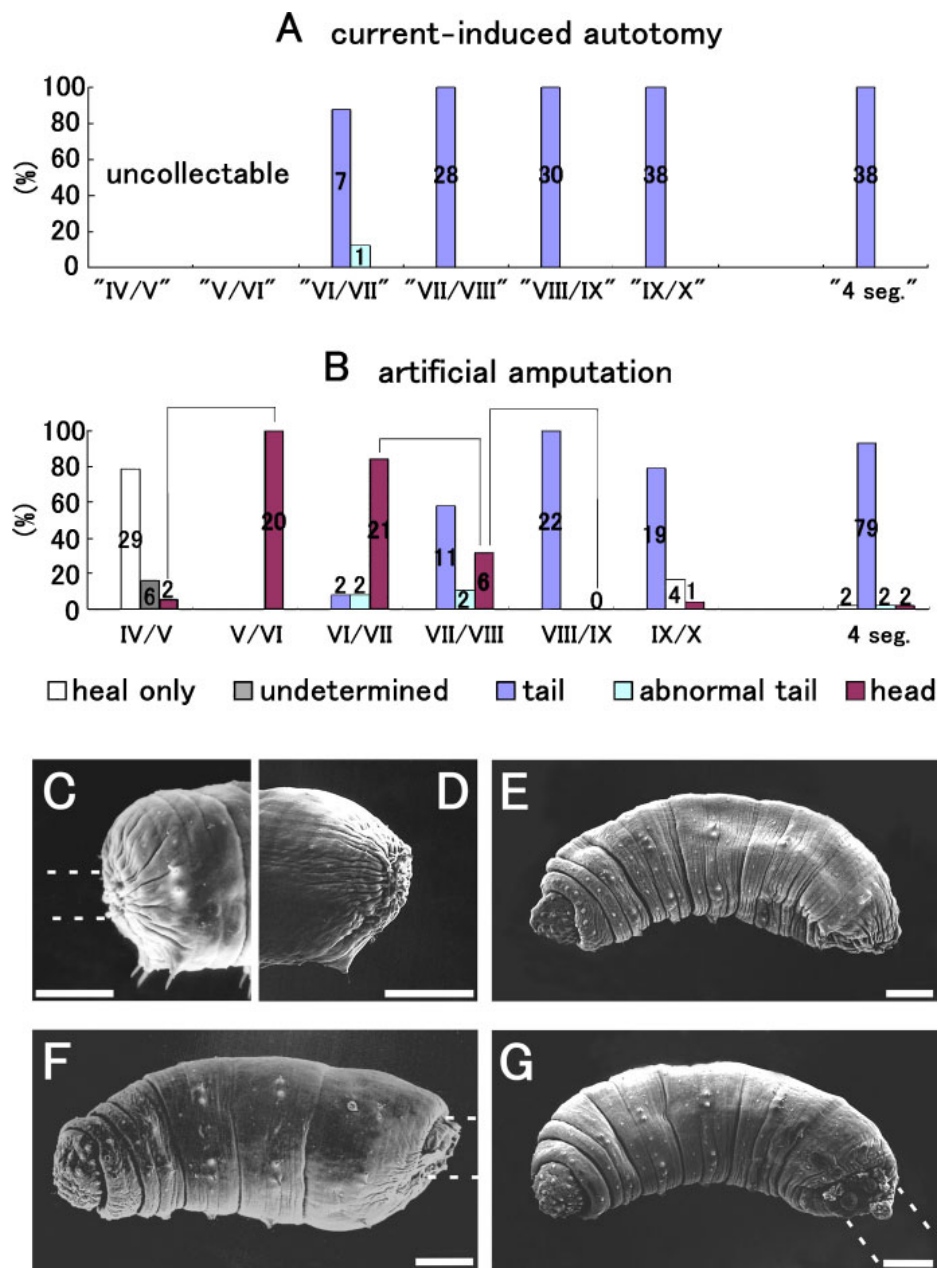


Fig. 5. Regeneration from head fragments. (A) Regeneration of autotomy fragments obtained by electrical stimulus. "VI/VII" indicates the fragments that had segments from the prostomium to VI and a tiny part of VII (they autotomized at the autotomic position in segment VII). No fragments shorter than fragments "VI/VII" were obtained. Basically, all of the fragments obtained by this method regenerated a normal tail posteriorly (blue bars). The frequencies of bipolar head regeneration did not depend on the position of the body at which autotomy occurred ( $P > 0.05$ ,  $\chi^2$ ). (B) On the other hand, the frequencies of bipolar head regeneration did depend on the position of the body at which amputation occurred ( $P < 0.001$ ,  $\chi^2$ ). IV/V indicates the fragments obtained by artificial amputation at the intersegment IV/V. Most IV/V fragments did not regenerate at all but merely healed (white bars and gray bars). V/V and VI/VII fragments regenerated the head posteriorly at very high rates (red bars). Fragments containing the trunk segments (VIII/IX and IX/X) regenerated the normal tail posteriorly. The brackets indicate significant statistical difference between the bipolar head regeneration in neighbor groups, as calculated by Ryan's method. (C) SEM image of the normal anterior autotomy plane in the trunk segment, just after autotomy. Although the circular muscle closes the wound, the anterior wound is not completely closed (dotted lines). (D) The normal posterior autotomy plane just after autotomy. The wound is completely closed. (E) The autotomized head fragment that had the prostomium to segment VI and the most anterior annulus of segment VII just after autotomy (i.e., "VI/VII" in A). The posterior wound is completely closed, as seen in (D). (F,G) The fragments amputated at the V/V (F) and VI/VII (G) intersegments just after amputation. In both cases, the wound is wide open (dotted lines). All photographs are lateral images.

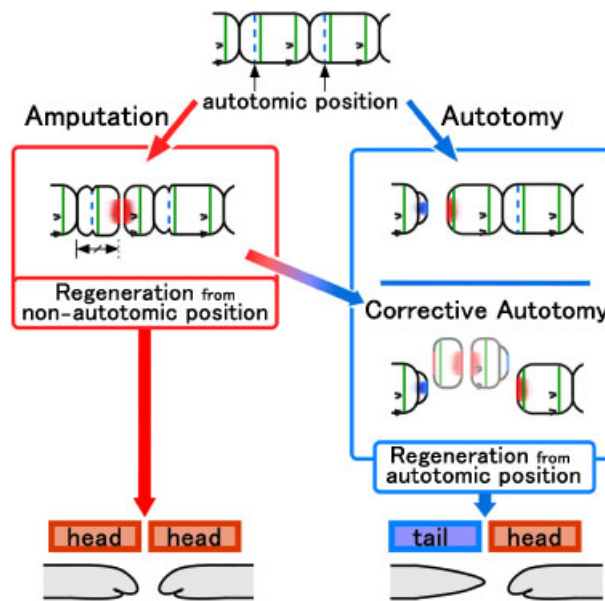


Fig. 6. Model for bipolar head regeneration. Normally, autotomy occurs at one specific position located anteriorly in each segment. When a worm is artificially amputated, the fragment correctively autotomizes spontaneously at the nearest autotomic position, then regenerates the head anteriorly and the tail posteriorly (normal regeneration). When corrective autotomy does not occur, a posterior head will be formed, resulting in a bipolar head worm. The bipolar arrow with a slash indicates the “incomplete short segment”, indicating that regeneration occurred from a non-autotomic position (cf. Fig. 1). The anterior amputation end always regenerates a head, regardless of whether corrective autotomy occurs or not.