

Research Articles: Systems/Circuits**Mosaic organization of body pattern control in the optic lobe of squids**Tsung-Han Liu¹ and Chuan-Chin Chiao^{1,2,3}¹*Institute of Molecular Medicine, National Tsing Hua University, Hsinchu, Taiwan*²*Institute of Systems Neuroscience, National Tsing Hua University, Hsinchu, Taiwan*³*Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan*

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38

39 **Abstract**

40 Cephalopods in nature undergo highly dynamic skin coloration changes that
41 allow rapid camouflage and intra-species communication. The optic lobe is thought to
42 play a key role in controlling the expansion of the chromatophores that generate these
43 diverse body patterns. However, the functional organization of the optic lobe and
44 neural control of the various body patterns by the optic lobe are largely unknown. We
45 applied electrical stimulation within the optic lobe to investigate the neural basis of
46 body patterning in the oval squid *Sepioteuthis lessoniana*. Most areas in the optic lobe
47 mediated predominately ipsilateral expansion of chromatophores present on the
48 mantle but not on the head and arms; furthermore, the expanded areas after electrical
49 stimulation were positively correlated with an increase in stimulating voltage and
50 stimulation depth. These results suggest a unilaterally dominant and vertically
51 converged organization of the optic lobe. Furthermore, analyzing fourteen of the
52 elicited body pattern components and their corresponding stimulation sites revealed
53 that the same components can be elicited by stimulating different parts of the optic
54 lobe and that various subsets of these components can be co-activated by stimulating
55 the same area. These findings suggest that many body pattern components may have
56 multiple motor units in the optic lobe and these are organized in a mosaic manner. The
57 multiplicity associated with the nature of the neural controls of these components in

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- 58 the cephalopod brain thus reflects the versatility of the individual components during
59 the generation of diverse body patterns.

60 **Significance Statement**

61 Neural control of the dynamic body patterning of cephalopods for camouflage
62 and intra-species communication is a fascinating research topic. Previous studies have
63 shown that the optic lobe is the motor command center for dynamic body patterning.
64 However, little is known about its neural organization and the mechanisms underlying
65 its control of body pattern generation. By electrically stimulating the optic lobe of the
66 oval squids and observing their body pattern changes, surprisingly, we found that
67 there is no somatotopic organization of motor units. Instead, many of these
68 components have multiple motor units within the optic lobe and that they are
69 organized in a mosaic manner. The present work reveals a novel neural control of
70 dynamic body patterning for communication in cephalopods.

71 **Introduction**

72 Cephalopods (octopus, squid, and cuttlefish) are a unique group of animals.
73 Their visual system and brain organization are the most sophisticated among all
74 invertebrates (Nixon and Young, 2003). Their dynamic color changes, which are used
75 for camouflage and intra-species communication, are an unparalleled feature of the
76 cephalopods (Hanlon and Messenger, 1996). These changes are the result of a special
77 sensorimotor system that is able to quickly change the animal's skin coloration via
78 neural control of numerous chromatophore organs within the dermis. Each
79 chromatophore organ is composed of an elastic sacculus that contains pigment
80 granules and is surrounded by radial muscles (Cloney and Florey, 1968). Expansion
81 and contraction of the chromatophore organ is thus mediated by these radial muscles,
82 which are in turn controlled by motor neurons running directly from the
83 chromatophore lobe in the brain without any synapses (Messenger, 2001).

84 Neural control of the dynamic body patterning of cephalopods is organized
85 hierarchically via a set of lobes within the brain; these are the optic lobes, the lateral
86 basal lobes and the anterior/posterior chromatophore lobes (Williamson and Chrachri,
87 2004). At the highest level, the optic lobes select specific motor commands, such as
88 body pattern or locomotion, which are largely based on visual input from the eyes. At
89 the intermediate control centers within the lateral basal lobes, there are abundant

90 projections from the optic lobes as well as large fiber tracts connecting to the
91 downstream lower motor centers, namely the anterior and posterior chromatophore
92 lobes (Boycott, 1961; Young, 1971; Young, 1974). Finally, each chromatophore
93 organ is innervated by more than one motor neuron from a chromatophore lobe and
94 one motor neuron controls multiple chromatophore organs (Dubas and Boyle, 1985).

95 The optic lobes are large, complex structures, each taking up one-third of the
96 total brain volume (Young, 1974) and they have a variety of functions. The outer
97 cortex, also called the deep retina (Cajal, 1917), contains visual analyzing systems
98 that process the input from the retina itself (Young, 1971). The central medulla is not
99 only a visual memory store, but also a higher motor center (Boycott, 1961; Young,
100 1971; Young, 1974). Cell bodies in the medulla are clumped together into
101 characteristic “cell-islands” that are surrounded by neuropil and there is no obvious
102 histological differentiation within the medulla (Young, 1962). The visual inputs to the
103 cell islands are feature detectors akin to those seen in the mammalian visual cortex
104 (Breidbach and Kutsch, 1995). In cuttlefish, direct electrical stimulation in the
105 medulla evoked various body patterns unilaterally or bilaterally, but stimulating the
106 lateral basal lobes and chromatophore lobes only elicited a uniform darkening, either
107 ipsilaterally or bilaterally (Boycott, 1961). In addition, electrical stimulation in the
108 medulla also produced various types of locomotive behavior (Chichery and Chanelet,

109 1976, 1978). These experiments are consistent with the hierarchical connections
110 found in the cephalopod brain and suggest that the medulla of the optic lobe is the
111 motor command center for dynamic body patterning.

112 Despite our overall understanding of optic lobe structure and function, little is
113 known about its neural organization and the mechanisms underlying its control of
114 body pattern generation. The aim of the present study is to examine the spatial
115 distribution of motor units that control the expression of body pattern components by
116 electrically stimulating various areas in the optic lobe of the oval squids *Sepioteuthis*
117 *lessoniana*. Surprisingly, there is no somatotopic organization of motor units in the
118 optic lobe. Instead, it was found that same components can be elicited by stimulating
119 different parts of the optic lobe and subsets of these components are able to be
120 co-activated by stimulating the same area. It is likely that many body pattern
121 components are controlled by multiple motor units and they are organized in the optic
122 lobe in a mosaic manner.

123 **Materials and Methods**124 **Animals**

125 Young adult oval squids of either sex, *Sepioteuthis lessoniana* (mantle length 4-12 cm)
126 were collected by a handheld fishing net from the waters off the northeast coast of
127 Taiwan. All animals were maintained in the laboratory using two close-circulation
128 aquarium systems (700 liters each; water temperature, 22~24°C). The room was kept
129 on a 12 hours light and 12 hours dark cycle. Before experimentation started, the
130 squids were anesthetized with magnesium chloride ($MgCl_2$, 100 mM) in cold seawater
131 (15-20 °C). Once animals ceased movement and their skins turned pale (Mooney et al.,
132 2010; Gleadall, 2013), they were transferred to a container supplied with circulating
133 oxygenated cold seawater (10-15 °C) at a reduced $MgCl_2$ concentration (50 mM) that
134 allowed prolonging sedation during electrical stimulation. To ensure the squids were
135 mildly anesthetized throughout the experiment, the animal's respiration rate, baseline
136 behavior, and coloration pattern was continuously assessed. This method has been
137 demonstrated to anesthetize the longfin squid (*Doryteuthis pealeii*) noninvasively for
138 up to 5 hours, and it has no apparent effect on physiological evoked potentials
139 recorded from nerve bundles within the statocyst system (Mooney et al., 2010). For
140 collecting the brain samples in histological analysis, the animals were not allowed to
141 recover from anesthesia, and were euthanatized by overdose of $MgCl_2$. All works

142 were carried out in accordance with the National Tsing Hua University guidelines, and
143 all procedures were approved by the institutional animal care and use committee.

144

145 **Electrical stimulation**

146 Oval squids are so transparent, thus we can access the optic lobe very
147 precisely without any surgery (Fig. 1). To stimulate the medulla of the optic lobe
148 efficiently, a bipolar electrode was used to provide electrical stimulation. Either a
149 custom-made electrode with a pair of coated tungsten wires (diameter, 50 μm) in a
150 27-gauge needle as a cannula or a commercial tungsten concentric electrode
151 (MicroProbes, USA) was used in the present study (Impedance 3-4 M Ω). A
152 stereotaxic device (Stoelting, USA) was used to assist the insertion of the electrode
153 and to map the stimulation sites with greater precision. Similar to the protocol
154 described in Boycott (1961), electrical stimulation was for 10 sec using 60 Hz pulses
155 (3 ms) at 3-10 volts and was delivered by an isolated pulse stimulator (AM2100, A-M
156 systems, USA). This 10 sec stimulation provided us with sufficient time to study
157 temporal dynamics of chromatophore expansion and interaction of body pattern
158 components. The minimum amount of stimulation voltage needed to elicit a
159 detectable skin response was 3 volts (or ~1 μA) in the present study. To ensure that
160 the electrical stimulation is confined in a small area, the current spreading examined

161 by measuring voltage drop at various distances from the stimulation site showed that
162 the electrical stimulation by using a bipolar electrode is indeed localized (Fig. 2).
163 These tests also demonstrated that the maximal stimulation voltage of 10 volts (~3 µA)
164 would not spread beyond a few tens of microns, and the likelihood of evoked skin
165 responses was arisen from stimulating a restricted area in the optic lobe. Either a LED
166 light or an audio device was used to signal when the electrical stimulation was turned
167 on and off; furthermore, a digital video camera (W100, Sony, Japan) was mounted
168 above to record the dynamic changes of body patterns that occurred. After each
169 successful electrical stimulation, the stimulation site was marked by
170 electrocoagulation with 1 mA current for 20 sec (Chichery and Chanelet, 1976), and
171 the optic lobe was isolated for histological analysis.

172

173 **Histological analysis**

174 All isolated optic lobes were fixed with 10% formaldehyde in seawater for 3
175 days and then transferred to 70% ethanol for further processing. To identify the
176 stimulation site in the medulla, the optic lobe was cryosectioned sequentially in the
177 sagittal plane using a cryostat (Leica, Germany). The optic lobe slices (30 µm in
178 thickness) were rinsed in 0.1 M phosphate buffer and mounted in glycerol for image
179 acquisition using a stereomicroscope (Zeiss, Germany). These images were then used

180 to map the stimulation sites in the 3-dimentional space of the optic lobe along three
181 major axes: the anterior-posterior axis (A-P axis), the medial-lateral axis (M-L axis),
182 and the depth axis. Note that the relative depth of electrical stimulation was
183 normalized relative to the total depth of the medulla. In this context, due to the
184 anatomical structure of the optic lobe, the total depth of the medulla was determined
185 in two different ways depending upon the location in the optic lobe (Fig. 3).

186

187 **Image analysis**

188 In the first set of image analysis, to quantify the body pattern change upon
189 electrical stimulation in the optic lobe, two images from each video recording were
190 acquired. One was the image frame immediately before the stimulus onset and the
191 other was the image frame when the animal showed the maximum response during
192 the 10 seconds stimulation. The maximum response was determined when the animal
193 showed the largest chromatophore expression during electrical stimulation. To
194 examine the chromatophore expansion in different parts of the body relative to the
195 stimulation site, each image was further divided into six regions: the ipsilateral mantle,
196 head, and arms as well as the contralateral mantle, head, and arms. The increase in
197 responsive area upon electrical stimulation in each region (i.e., the chromatophore
198 expansion) was quantified by calculating the relative area increase the pixel values of

199 which were above the threshold in the image after stimulation using Image J (NIH,
200 USA). The thresholds were set as a 10%, a 20%, a 30%, or a 40% increase in pixel
201 intensity relative to the mean intensity in the image before stimulation. In the present
202 study, only the results with a 20% threshold are shown (i.e., pixels whose intensity
203 changed more than 20% were summed to give a total area), but the other thresholds
204 showed a similar trend. To evaluate the lateralization of body pattern control by the
205 optic lobe, a lateralization index was calculated by taking the normalized difference in
206 increased responsive areas between ipsilateral and contralateral sides as:
207 $[Ipsi-Contra]/[Ipsi+Contra]$.

208 In the second set of image analysis, to further assess the expression of
209 individual body pattern components upon electrical stimulation, we identified
210 fourteen distinct chromatic components in *S. lessoniana* based on numerous fields and
211 laboratory images (Fig. 4). Some of these components are similar to the ones
212 described in *Sepioteuthis australis* (Jantzen and Havenhand, 2003a, b), but there are a
213 few unique components in *S. lessoniana* (the ethogram and related data will be
214 published separately). The expression level of these components was graded manually
215 using images after stimulation. The grading scheme followed the one used in
216 quantifying component expression in camouflaged cuttlefish (Mathger et al., 2006).
217 Each component was assigned one of number of grades, namely 0 (not expressed), 1

218 (weakly expressed), 2 (moderately expressed) and 3 (strongly expressed); the levels of
219 expression were graded separately for the ipsilateral and contralateral sides.

220

221 **Statistical analysis**

222 All data were analyzed using t-tests or using one-way repeated measures

223 ANOVA (SPSS, USA) with Tukey *post-hoc* analysis for pairwise comparisons.

224

225 **Results**

226 Expansion of chromatophores and expression of body pattern components
227 were frequently observed when electrical stimulation was applied within the medulla
228 of the optic lobe (Fig. 5). The speed of skin coloration change was often swift (i.e.,
229 within 1-3 sec of electrical stimulation onset in these anesthetized animals), and some
230 body pattern components were elicited faster than others (data not shown). However,
231 not all stimulations with the same protocol elicited a skin coloration change. In more
232 than 100 attempts at various locations in the optic lobe, only 65 stimulations from
233 different animals had distinct evoked responses. Although the exact reason for these
234 failed trials is not known, it is apparent that the optic lobe became less responsive to
235 electrical stimulation when the normal circulation was reduced during prolonged
236 anesthesia. These successful stimulation sites were distributed randomly in the optic
237 lobe (Fig. 6). The maximal change in evoked responses and expression of individual
238 body pattern components were analyzed along with their corresponded stimulation
239 sites in order to investigate the neural organization of body pattern control within the
240 optic lobe.

241

242 **Control of chromatophore expression in the optic lobe is ipsilaterally dominant**
243 **and non-somatotopically organized**

244 To examine whether chromatophore expression is controlled ipsilaterally or
245 contralaterally in the optic lobe, the increased responsive areas (i.e., chromatophore
246 expansion) upon electrical stimulation in mantle, head, and arms were quantified and
247 the lateralization indices were calculated. It is apparent that stimulating the optic lobe
248 elicited more ipsilateral expression of chromatophores on the mantle than on the head
249 and arms (Fig. 7). Although the majority of stimulations evoked more ipsilateral
250 expansion of chromatophores on the mantle, there were some instances where there
251 was contralateral and bilateral control. This was more evident when the responsive
252 areas were the head and arms. Nevertheless, considering that the mantle makes up
253 about 80% of body area, the control of chromatophore expression in the optic lobe
254 remains still ipsilaterally dominant.

255 To further determine whether control of chromatophore expression is
256 organized somatotopically in the optic lobe, the increases in expression areas on the
257 ipsilateral mantle, head, and arms upon electrical stimulation in anterior, middle, and
258 of the posterior optic lobes were compared. Regardless of the position of the
259 stimulation sites in the optic lobe, chromatophore expression on the mantle showed
260 the largest increases (Fig. 8). This suggests that control of chromatophore expression
261 along the body axis shows no topographic correspondence to the optic lobe and that
262 the mantle appears to undergo significantly more control than the head and arms

263 within the high motor center of the brain.

264

265 **Chromatophore expansion is determined by activation strength and depth in the
266 optic lobe**

267 To assess the effect of activation strength on the control of chromatophore
268 expression, increases in ipsilateral expression area upon electrical stimulation were
269 compared between two stimulation strengths (X and X+2 volts) at the same site in the
270 optic lobe. It is clear that when the stimulation voltage was increased by 2 volts, the
271 expression area was increased significantly (Fig. 9A). This suggests that a stronger
272 stimulation activates more of the units controlling chromatophore expression in the
273 optic lobe, thus increasing the expressed area. However, increasing the stimulation
274 voltage by 2 volts had no significant effect on the bilateral chromatophore expression
275 response (Fig. 9B). This implies that activating more chromatophore expression
276 control units in the optic lobe does not necessary increase the level of crossover in
277 terms of motor commands to the contralateral side.

278 Similarly, to examine the effect of activation depth on the control of
279 chromatophore expression, the increases in ipsilateral expression area upon electrical
280 stimulation were compared between two stimulation depths (X and X+1 mm) in the
281 optic lobe using the same stimulation strength. It became evident that when the

282 stimulation depth was increased, the expression area was increased significantly (Fig.
283 10A). This suggests that deeper stimulation activates more chromatophore expression
284 control units in the optic lobe, thus increasing the expressed area. However, increasing
285 the stimulation depth by 1 mm had no significant effect on the bilateral
286 chromatophore expression response (Fig. 10B). This implies that activating more
287 chromatophore expression control units in the optic lobe does not necessarily increase
288 the crossover of motor commands into the contralateral side.

289

290 **The motor units of individual body pattern components are multiplexed and
291 interconnected within the optic lobe**

292 It is known that cephalopod body patterns can be analyzed into their
293 components (Packard and Hochberg, 1977), and these components can be thought of
294 not only as morphological units within the skin but also as physiological units within
295 the brain (Packard, 1982). To understand how the optic lobe organizes the motor units
296 of individual body pattern components and ultimately generates an appropriate body
297 patterns for communication, the expression levels of fourteen body pattern
298 components from the dorsal and lateral sides of the squid (Fig. 4) upon electrical
299 stimulation were analyzed. Each component expression level on the ipsilateral and
300 contralateral sides was graded separately (ranging from 0 to 3, see Methods for

301 details). For example, both “Dark head” and “Mottle fins” can be activated by
302 stimulating the optic lobe at multiple sites along the medial-lateral axis, although the
303 sites that elicit “Dark head” are more widespread than those of evoking “Mottle fins”,
304 and the components were expressed more bilaterally with regard to “Dark head” than
305 regarding “Mottle fins” (Fig. 11). Comparing the lateralization index of all fourteen
306 body pattern components, it is apparent that most mantle-related components have
307 higher lateralization indices, while head/arms-related components tended to have
308 lower indices (Fig. 12). Specifically, ten body pattern components showed
309 significantly ipsilateral dominance, including seven mantle-related components and
310 three head/arms-related components. The results are consistent with our finding that
311 stimulation of the optic lobe elicited more ipsilateral expression of chromatophores on
312 the mantle than on the head and arms. This also suggests that, within the optic lobe,
313 body pattern components on the mantle are controlled more ipsilaterally, whereas
314 those on the head and arms are controlled more bilaterally.

315 To see the spatial relationship of motor units that control the expression of
316 individual components within the optic lobe, all stimulation sites responsible for
317 evoking the fourteen body pattern components were visualized separately using 3D
318 plots (Fig. 13). The results clearly show that some components were evoked
319 frequently (e.g., Dark mantle), while others were encountered much less often (e.g.,

320 Fin margin spots). Nevertheless, most body pattern components are able to be evoked
321 by stimulating multiple sites in the optic lobe and these sites are widely distributed.
322 These findings suggest that individual components may have multiple motor units,
323 and they are organized nonsomatotopically, or perhaps at least diffusely in the optic
324 lobe. Further analysis revealed that some components are able to be co-activated by
325 stimulating the same area in the optic lobe. To assess the spatial organization of motor
326 units responsible for component activation, a joint probability of co-expression of two
327 components was computed for each of the fourteen components (Table 1). For
328 example, when stimulating the same site, if “Dark mantle” is evoked, then “Dark
329 arms” is also evoked with a 94% probability, but “Fin margin spots” is evoked in 0%
330 probability. To visualize this table, the distribution of the evoked probabilities of
331 fourteen components and their co-expression probabilities with other components
332 were shown as multiple pie charts (Fig. 14). Each of the surrounding pie charts
333 depicts the distribution of joint-probabilities of each component with the other
334 thirteen components. It is clear that subsets of the components are able to be
335 co-activated by stimulating the same area with different joint-probabilities. This
336 suggests that the motor units of individual components may be clustered in the optic
337 lobe and they may be organized in a mosaic manner.

338 While stimulating weakly anesthetized animals in the present study was for a

339 practical reason, we have attempted to stimulate the optic lobe of quasi-aware squids
340 (i.e., they had 5-10 min recovery from anesthesia, and restored normal ventilation and
341 showed vigorous fin movement) while the electrode was still intact. Although this is
342 not equivalent to fully aware and free swimming animals, this additional experiment
343 confirms that electrical stimulation of the optic lobe in anesthetized and quasi-aware
344 animals evokes similar body pattern components (Table 2 and Fig. 15). Note that in
345 some cases a few additional components not seen in the anesthetized squids were
346 elicited in the quasi-aware squids (Table 2), and even the yellow chromatophores
347 could sometimes be evoked during recovery (Fig. 15). It is likely a result of increased
348 spontaneous neural activity and other sensory inputs in the quasi-aware state.
349 Nevertheless, this observation reveals that the strength and speed of skin patterning
350 response in the quasi-aware animals generally match what is seen in the anesthetized
351 animals. This also suggests that the mosaic organization of body pattern control in the
352 optic lobe derived in the present study largely holds true even with other sensory input
353 and neural activity in awake animals. Finally, to confirm the joint-probabilities of
354 each component with the other thirteen components in live animals, the co-expression
355 of components on squids were analyzed (Fig. 16). Squids with different behaviors
356 show distinct body patterns that are composed of different components. Indeed,
357 co-activated components have a higher joint-probability of co-expression and this has

358 been clearly revealed in the present study.

359

360 **Discussion**

361 **Control of body coloration is ipsilaterally dominant and nonsomatotopically**

362 **organized in the optic lobe**

363 The optic nerve fibers from each eye are projected to the ipsilateral optic lobe

364 in a regular pattern, being precisely re-assorted after passing through a chiasma that

365 maintains a retinotopic organization in the cortex of the optic lobe (Young, 1962,

366 1974). The optic tracts from each optic lobe are then projected to many areas in the

367 central brain, as well as the contralateral optic lobes via the commissure. Consistent

368 with a previous study showing that direct electrical stimulation within the optic lobe

369 evoked various body patterns unilaterally or bilaterally in cuttlefish (Boyce, 1961),

370 the present study also found that stimulating the optic lobe of squids on one side

371 elicited selective darkening on both sides to a variety of extents. However, our results

372 also revealed that stimulation of the optic lobe evoked more ipsilateral expression of

373 chromatophores on the mantle than on the head and arms (Fig. 7). Further analysis

374 also indicated that expression of body pattern components on the mantle showed

375 significantly ipsilateral control, whereas expression of body pattern components on

376 the head and arms showed more bilaterally controlled (Fig. 12). Taking into

377 consideration the fact that the mantle makes up a much larger area than the head and

378 arms in squids, the presence of color changes on the mantle with a significantly

379 degree of ipsilateral control suggests that the motor control of body patterning is
380 largely dependent on visual input from the ipsilateral side. The evidence that the head
381 and arms are more bilaterally controlled may indicate that the commissure for
382 regulating these areas is enhanced or these areas require symmetric expression for
383 effective visual communication.

384 Many of the optic tracts leaving the optic lobes maintain a precisely
385 topographical organization in some motor centers (e.g., peduncle lobe) and the
386 contralateral optic lobe via the commissure. However, anatomical studies also have
387 found that the output projection for the control of body coloration from the optic lobe
388 shows complex interweaving and the response pattern is not topographically related to
389 the visual input (Young, 1974). Our findings indicate that the control of
390 chromatophore expression along the body axis shows no topographic correspondence
391 to the optic lobe (Fig. 8) and that most body pattern components are able to be evoked
392 by stimulating multiple sites with these sites being widely distributed across the optic
393 lobe (Fig. 13). This suggests that the motor output of the optic lobe for body pattern
394 control does not correspond to the animal's visual input and there is no somatotopic
395 mapping of motor output across the optic lobe. Instead, chromatophore expression of
396 the skin is controlled across the entire optic lobe. This is also consistent with a
397 previous study showing that discrete and complex components of arm movements

398 have no central topographical organization in the higher motor center, but are rather
399 distributed over wide regions in the octopus (Zullo et al., 2009). Furthermore, tracer
400 injection of chromatophores on the skin and electrical stimulation at the posterior
401 chromatophore lobe in squids have also demonstrated that there is no relationship
402 between the loci in the lower motor center and regions of chromatophore expansion at
403 the periphery (Dubas et al., 1986b; Dubas et al., 1986a). Taken together, our findings
404 support the hypothesis that motor control in cephalopods is non-somatotopically or
405 diffusely organized in the brain. This is a sharp contrast of vertebrates where
406 topological organization and sensory motor mapping underlie much of our
407 understanding about functional organization and development.

408

409 **The motor units of body pattern components are organized in a mosaic pattern**
410 **in the optic lobe**

411 The body pattern of cephalopods is a combination of chromatic, textural,
412 postural, and locomotor components and a subset of these components may be
413 combined together at any time to create a given different body pattern (Packard and
414 Sanders, 1969; Packard and Sanders, 1971; Packard and Hochberg, 1977; Hanlon and
415 Messenger, 1996). More importantly, these components can be thought of not only as
416 morphological units of the body but also as physiological units within the brain

417 (Packard, 1982). To focus on the neural control of chromatophore expression in the
418 skin, only chromatic components were considered in the present study. Although the
419 body pattern components in *S. lessoniana* have not been characterized systematically,
420 those in other oval squid species are well documented (Moynihan and Rodaniche,
421 1982; Jantzen and Havenhand, 2003b). Following these earlier component
422 nomenclatures, we have defined fourteen distinct chromatic components in young
423 adult *S. lessoniana* as part of the present study. If these fourteen components represent
424 distinct physiological units in the brain, our results suggest that each of these
425 components does not reside in a single locus within the optic lobe, but rather each can
426 be evoked by stimulating multiple sites in the medulla (Fig. 13). However, the
427 frequency of each component that can be elicited by electrical stimulation was
428 different (Fig. 14). Among them, Dark arms (15.5%), Dark mantle (14.9%), Dark
429 tentacles (13.9%), and Dark head (9.7%) were the most frequently encountered ones.
430 These components, each of which has a different level of expression, represent a
431 uniform body pattern, which is one of the most commonly seen body patterns in the
432 wild. The less frequently evoked components, including Fin margin spots and Dark
433 fin margin, are rarely seen in young adult squids, but are often observed in sexually
434 mature animals during the reproductive season. These findings suggest that some
435 motor units of components may be added to the optic lobe later in life in order to

436 expand the repertoire of body patterning.

437 In addition to finding that individual components are able to be elicited by

438 stimulating multiple sites in the medulla of the optic lobe, we also found that a subset

439 of components can often be co-activated by the same stimulation (Fig. 14). The

440 expression level of each evoked component was not the same and the joint-probability

441 of co-expression varied among the different components (Table 1). These

442 observations indicate that the motor units of the body pattern components are not only

443 repeatedly present, but are also dispersedly distributed within the optic lobe. This

444 neural organization may allow squids to generate diverse body patterns by selectively

445 activating a subset of components. To conceptualize this optic lobe control of body

446 patterning, we propose a mosaic organization model in which the motor units of

447 individual components are organized in a mosaic fashion within a motor command

448 module for body pattern generation (Fig. 17A). Each module may contain all the

449 control units of components, each with a different proportion (as represented by

450 different color circles in Fig. 17A), and these modules are likely to occur multiple

451 times in the medulla of the optic lobe. Thus, when stimulating any module in the optic

452 lobe, various different numbers of components can be evoked. In turn, different body

453 patterns can be generated by activating distinct sub-regions in the module. We

454 speculate that the structural basis of these modules may be the reported cell islands in

455 the medulla (Young, 1974). Based on the histological analysis, all stimulation sites but
456 two were found to be located in the cell islands, not in the neuropils. This supports
457 that the cell islands are likely the proposed motor command modules. Even the only
458 two neuropil stimulations also generated similar response, which suggests that the
459 neural fibers leaving the cell islands carry similar motor command information. In
460 addition, magnetic resonance imaging (MRI) evidence has demonstrated that the cell
461 islands are continuous structures in the optic lobe (unpublished data), and the area of
462 these islands gradually increases toward the optic tract. This anatomical observation
463 corroborates our results that chromatophore expansion upon electrical stimulation is
464 positively correlated with an increase in stimulating voltage and a greater depth of
465 stimulation (Figs. 9 and 10). Moreover, not only are the expression areas expanded, in
466 some cases we also observed that the number of evoked body pattern components was
467 increased (but never decreased) when the stimulating voltage was increased and when
468 there was a greater depth of stimulation. This further supports the mosaic model of the
469 body pattern control in the optic lobe. However, due to the persistence of our
470 electrical stimulation (10 sec), there may be some cumulative effect in the optic lobe
471 or in the downstream projections to lower motor centers, thus this mosaic model
472 requires further temporal analysis of body pattern component expression. The
473 distribution of the motor units of these frequently evoked components within the

474 module is somewhat random (Fig. 17A), thus they can be activated often by electrical
475 stimulation. However, the motor units of less often encountered components are
476 positioned near the motor units of the components with a higher joint-probability of
477 co-expression (e.g., Dark fin margin in Fig. 17A), thus they can be co-activated
478 together to produce certain body patterns. Future studies with more targeted
479 stimulation will help to verify the exact distribution and interaction of the control
480 units of individual body pattern components in the module.

481 Although this mosaic organization of neural control units of body patterning in
482 the optic lobe is seemingly redundant, it does provide an efficient way of coordinating
483 expression of the multiple components needed to generate diverse body patterns (a
484 diagram summarizing the visual motor system of body pattern control is shown in Fig.
485 17B). This is similar to muscle control during locomotion in vertebrates, in which
486 each muscle can be activated by stimulating many widely dispersed sites in the motor
487 center in order to coordinate whole-limb actions (Ting and McKay, 2007). The
488 concurrent activation of synergies thus simplifies the neural command signals needed
489 for movement, while allowing flexibility and adaptability. Finally, the multiplicity and
490 arrangement of these modules in the medulla of the optic lobe may represent a
491 complex extensive repeated organization of the motor commands. The network of
492 lateral and vertical connections among these modules is likely to provide a

493 mechanism allowing sensorimotor integration of dynamic body patterning in

494 cephalopods.

495

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503

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- 568

569

570

Table 1. Joint probability of co-expression of two body pattern components

	DM	MB		MF		DFM		FMS		DMS		DLS	
571 DA	0.94	DM	0.63	DM	0.96	DM	1.00	MB	1.00	DM	0.89	DA	0.89
572 DT	0.80	DH	0.50	DA	0.88	MF	1.00	DH	1.00	DA	0.86	DM	0.83
573 DMS	0.70	DA	0.50	DT	0.83	MH	1.00	DM	0.00	DT	0.81	DT	0.83
574 DH	0.52	DT	0.50	DMS	0.71	DA	1.00	MF	0.00	DH	0.56	DMS	0.72
575 MF	0.50	MF	0.38	DH	0.50	DT	0.67	DFM	0.00	MF	0.47	DH	0.50
576 DLS	0.33	DMS	0.38	SE	0.38	MB	0.33	DMS	0.00	DLS	0.36	ES	0.39
577 MH	0.28	MMS	0.25	MH	0.33	MMS	0.33	DLS	0.00	MMS	0.31	MF	0.33
578 MMS	0.26	ES	0.25	ES	0.29	FMS	0.00	MMS	0.00	SE	0.28	MMS	0.28
579 ES	0.26	DFM	0.13	DLS	0.25	DMS	0.00	ES	0.00	ES	0.25	MH	0.28
580 SE	0.26	FMS	0.13	MMS	0.25	DLS	0.00	MH	0.00	MH	0.22	SE	0.17
581 MB	0.11	MH	0.13	MB	0.13	ES	0.00	SE	0.00	MB	0.08	MB	0.00
582 DFM	0.07	SE	0.00	DFM	0.13	DH	0.00	DA	0.00	DFM	0.00	DFM	0.00
583 FMS	0.00	DLS	0.00	FMS	0.00	SE	0.00	DT	0.00	FMS	0.00	FMS	0.00
	MMS	ES		DH		MH		SE		DA		DT	
584 DM	0.86	DA	1.00	DA	0.87	DM	1.00	DM	1.00	DM	0.90	DA	0.93
585 DMS	0.79	DM	0.92	DT	0.87	DA	1.00	DA	0.92	DT	0.83	DM	0.86
586 DA	0.79	DT	0.77	DM	0.80	DT	0.69	DMS	0.83	DMS	0.65	DMS	0.67
587 DT	0.79	DMS	0.69	DMS	0.67	MF	0.62	DH	0.83	DH	0.54	DH	0.61
588 DH	0.57	MF	0.54	MF	0.40	DMS	0.62	DT	0.83	MF	0.44	MF	0.47
589 MF	0.43	DLS	0.54	SE	0.33	DLS	0.39	MF	0.75	DLS	0.33	DLS	0.35
590 DLS	0.36	DH	0.54	DLS	0.30	MMS	0.39	MMS	0.33	ES	0.27	MMS	0.26
591 MH	0.36	SE	0.32	MMS	0.27	DFM	0.23	ES	0.33	MH	0.27	ES	0.23
592 SE	0.29	MH	0.23	ES	0.23	ES	0.23	DLS	0.25	MMS	0.23	SE	0.23
593 MB	0.14	MB	0.15	MB	0.13	SE	0.15	MH	0.17	SE	0.23	MH	0.21
594 DFM	0.07	DFM	0.00	FMS	0.03	MB	0.08	MB	0.00	MB	0.08	MB	0.09
595 FMS	0.00	FMS	0.00	DFM	0.00	FMS	0.00	DFM	0.00	DFM	0.06	DFM	0.05
596 ES	0.00	MMS	0.00	MH	0.00	DH	0.00	FMS	0.00	FMS	0.00	FMS	0.00

597 DM: dark mantle, MB: mantle bands, MF: mottle fins, DFM: dark fin margin, FMS: fin margin spots, DMS: dark mantle stripe, DLS: dark longitudinal

598 stripe, MMS: mantle margin stripe, ES: eye spots, DH: dark head, MH: mottle head, SE: shaded eyes, DA: dark arms, DT: dark tentacles.

591 **Table 2. Body pattern components evoked by electrically stimulating the optic**
592 **lobe of anesthetized and quasi-aware oval squids**

Trial	Anesthetized animal	Quasi-aware animal
1	DM, DMS, DH, DA, DT	DM, DMS, DH, DA, DT
2	DM, DMS, DLS, DH, DA, DT	DM, DMS, DLS, DH, DA, DT
3	DLS, DH, DA, DT	DLS, DH, DA, DT, DM, DMS
4	DM, MB, MF, DA, DT	DM, MB, MF, DA, DT
5	DM, DMS, DH, DA, DT	DM, DMS, DH, DA, DT, MF
6	DM, MB, DLS, SE, DA, DT	DM, MB, DLS, SE, DA, DT
7	DM, DMS, DLS, DH, DA, DT	DM, DMS, DLS, DH, DA, DT, MF, ES

593 *DM: dark mantle, MB: mantle bands, MF: mottle fins, DMS: dark mantle stripe,
594 DLS: dark longitudinal stripe, ES: eye spots, DH: dark head, SE: shaded eyes, DA:
595 dark arms, DT: dark tentacles.

596

597

598

599 **Figures and figure legends**

600 **Figure 1.** The optic lobe of the oval squid, *Sepioteuthis lessoniana*. (A) A live
601 specimen. Dashed outlines depict a pair of optic lobes located behind the eyes. (B) A
602 schematic diagram showing the position of the optic lobe (gray area) relative to the
603 eye in the lateral view of the oval squid. (C) A dissected left optic lobe. The picture
604 shows the medial view of the optic lobe, where the site of the optic tract can be seen
605 (dented area). A, anterior; P, posterior; D, dorsal; V, ventral. Scale bars, 1 cm in (A)
606 and 0.5 cm in (C).

607

608 **Figure 2.** Electrical stimulation in the medulla of the optic lobe is localized. (A) The
609 recorded voltage responses decreased significantly as the distance from the
610 stimulation site increased (400 μ m, 800 μ m, and 1200 μ m) for various stimulation
611 voltages used in the present study. (B) The stimulating current was significantly
612 reduced at the site 400 μ m away from the stimulation site for various stimulation
613 voltages (400 μ m, N=16; 800 μ m, N=16, 1200 μ m, N=4). Error bars are SEM.

614

615 **Figure 3.** The depth of the electrical stimulation site in the optic lobe. Two
616 parasagittal sections of the optic lobe. (A) The optic lobe near the medial side,
617 showing the optic tract (asterisk). (B) The optic lobe near the lateral side. Both

618 sections show clearly the cortex and medulla of the optic lobe. To calculate the
619 relative depth of the stimulation site (red dot), the center of the medulla (yellow dot)
620 is determined either using the inner border of the optic lobe as in (A) or using the
621 central mass of the optic lobe as in (B). The blue line perpendicular to the cortex of
622 the optic lobe indicates the total depth. The relative depth of the stimulation site is
623 thus defined as travel distance of the electrode from the cortex divided by the total
624 depth. Note that the optic lobe in panel B apparently has two zones of cell islands, and
625 this is a result of concave surface of the optic lobe at the lateral side. Scale bar, 1000
626 μm .

627

628 **Figure 4.** Body pattern components of the oval squid, *Sepioteuthis lessoniana*. A total
629 of fourteen distinct body pattern components observed; these are depicted for the
630 present study in these drawings. Except for Dark longitudinal stripe and Mantle
631 margin stripe, which are shown in a lateral view, all the other twelve components are
632 shown in a dorsal view.

633

634 **Figure 5.** A subset of body pattern components that are evoked by electrically
635 stimulating the optic lobe of the oval squid. The time series images show the
636 expression of four distinct body pattern components (Mantle bands, Dark mantle

637 stripe, Dark head, and Dark arms) after the stimulation onset. Scale bar, 3 cm.

638

639 **Figure 6.** Stimulation sites in the 3D space of the optic lobe. (A) Total of 65
640 stimulation sites in the present study are shown along three major axes in the optic
641 lobe, including the anterior-posterior (A-P), medial-lateral (M-L), and depth. (B)-(D)
642 Projections of the 3D plot onto three 2D planes. The dotted outline in panel B
643 indicates the medulla area of the optic lobe that is accessible to the electrical
644 stimulation. This was estimated by measuring the relative size of the medulla using a
645 series of consecutive histological slices collected from the medial side to the lateral
646 side.

647

648 **Figure 7.** Stimulation of the optic lobe elicits more ipsilateral expression of
649 chromatophores on the mantle than on the head and arms. The lateralization index is
650 used to assess the symmetry of chromatophore expression upon electrical stimulation
651 in the optic lobe. Positive indices indicate ipsilateral dominance, and negative indices
652 show contralateral dominance. Chromatophore expression on the mantle showed
653 significantly ipsilateral control, whereas chromatophore expression on the head and
654 arms was more variable. ** $p<0.01$.

655

656 **Figure 8.** The control of chromatophore expression along the body axis has no
657 topographic correspondence in the optic lobe. Stimulation of various optic lobe
658 regions evoked different ipsilateral responses. (A) Anterior stimulation (first 1/3 of the
659 optic lobe). (B) Middle stimulation (middle 1/3 of the optic lobe). (C) Posterior
660 stimulation (last 1/3 of the optic lobe). Regardless the stimulation site, chromatophore
661 expression on the mantle showed the largest increase. Ipsi-mantle, ipsilateral mantle;
662 Ipsi-head, ipsilateral head; Ipsi-arms, ipsilateral arms. Error bars are SEM. * $p<0.05$
663 and ** $p<0.01$.

664

665 **Figure 9.** An increase in the stimulation voltage enhances ipsilateral chromatophore
666 expression, but not a bilateral response. (A) An increase in the stimulation voltage by
667 2 V (from X to X+2 V) enlarged the expression area of the chromatophores. (B) An
668 increase in the voltage had no significant effect on the bilateral response of
669 chromatophore expression. * $p<0.05$.

670

671 **Figure 10.** A greater depth of stimulation enhances ipsilateral chromatophore
672 expression, but not a bilateral response. (A) A greater depth of stimulation by 1 mm
673 (from X to X+1 mm) enlarges the expression area of the chromatophores. (B) A
674 greater depth of stimulation has no significant effect on the bilateral response of

675 chromatophore expression. * $p<0.05$.

676

677 **Figure 11.** The control of the expression of body pattern components is not confined
678 to a specific area of the optic lobe. Each elicited component was scored manually
679 based on the extent of the expression (0 means no expression and 3 means full
680 expression) and the side of the expression (positive means ipsilateral and negative
681 means contralateral). (A) The Dark head expression was evoked by stimulating wide
682 spread locations along the medial-lateral axis in the optic lobe, and its activation was
683 more bilaterally dominant. (B) The Mottle fins expression was elicited by stimulating
684 slightly more restricted areas in the optic lobe, and the response was less bilaterally
685 dominant.

686

687 **Figure 12.** Body pattern components on the mantle are controlled more ipsilaterally
688 within the optic lobe, whereas those on the head and arms are controlled more
689 bilaterally. The lateralization index is used to assess the symmetry of body pattern
690 component expression upon electrical stimulation in the optic lobe. Positive indices
691 indicate ipsilateral dominance, and negative indices show contralateral dominance.
692 Expression of body pattern components on the mantle showed significant ipsilateral
693 control, whereas those on the head and arms showed more bilateral controlled.

694 Numbers in parentheses indicate the sample size. * $p<0.05$ and ** $p<0.01$.

695

696 **Figure 13.** The stimulation sites of the fourteen body pattern components in the 3D
697 space of the optic lobe. The stimulation sites of each body pattern component in the
698 present study are shown along the three major axes in the optic lobe, including the
699 anterior-posterior (A-P), medial-lateral (M-L), and depth. Some components were
700 evoked often (e.g., Dark mantle), but some were encountered much less often (e.g.,
701 Fin margin spots).

702

703 **Figure 14.** Distribution of the evoked probabilities of the fourteen body pattern
704 components and their co-expression probabilities with other body pattern components.
705 The central pie chart shows the distribution of evoked probabilities of the fourteen
706 body pattern components in the present study. The surrounding pie charts are the
707 distribution of joint-probabilities of each body pattern component with other thirteen
708 components.

709

710 **Figure 15.** Electrical stimulation of the optic lobe in anesthetized and quasi-awake
711 animals evokes similar body pattern components. (A) Anesthetized animal before
712 stimulation. (B) The oval squid showed six distinct body pattern components (DM,

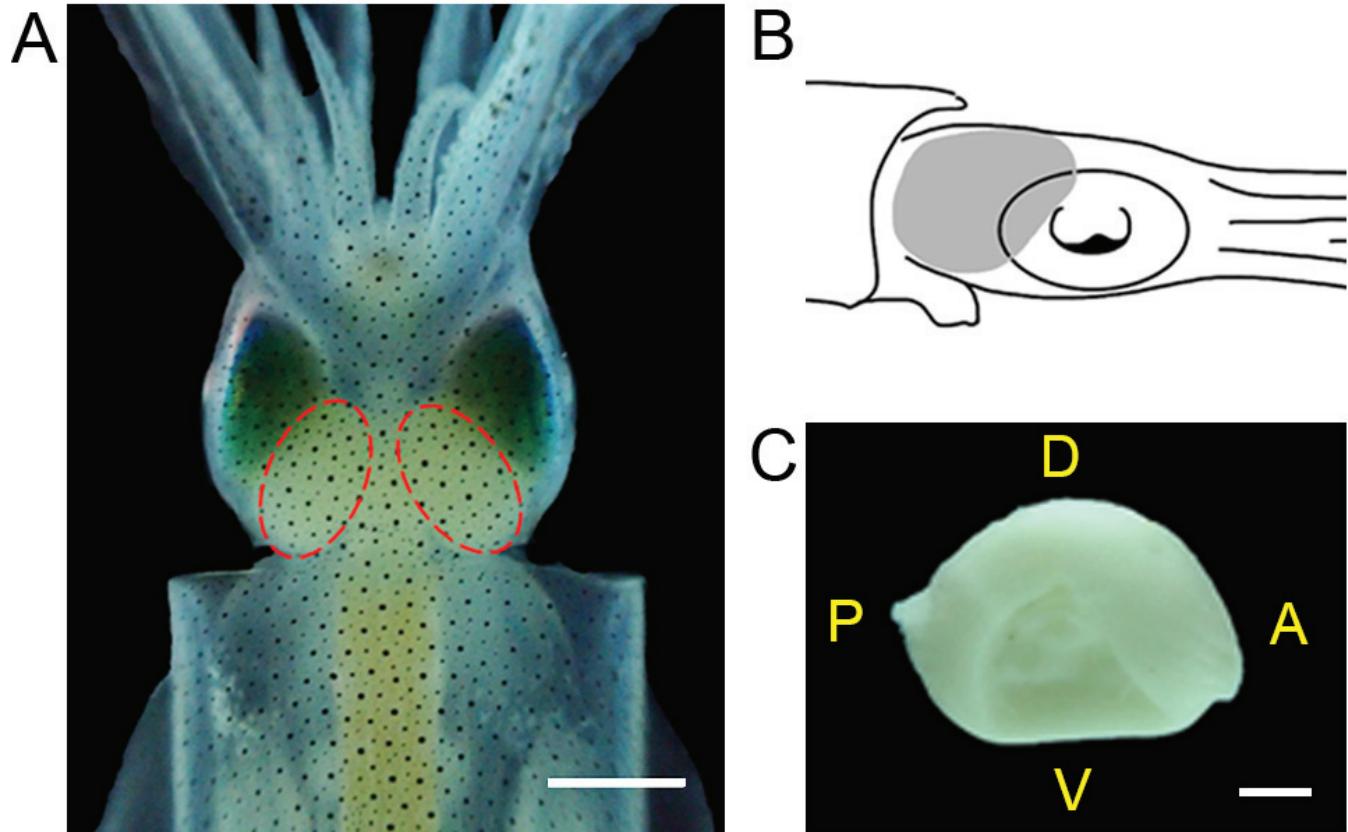
713 DMS, DLS, DH, DA, and DT) during electrical stimulation. (C) After 5 minute
714 recovery from anesthesia, the animal restored normal ventilation. (D) This half-awake
715 oval squid showed identical but stronger body pattern components when the same
716 electrical stimulation was applied again. (E) After 6 minute recovery from anesthesia,
717 the animal displayed fin movement. (F) The quasi-awake oval squid showed
718 additional two body pattern components (MF and ES) when the same electrical
719 stimulation was applied again. DM: dark mantle, DMS: dark mantle stripe, DLS: dark
720 longitudinal stripe, DH: dark head, DA: dark arms, DT: dark tentacles, MF: mottle
721 fins, ES: eye spots.

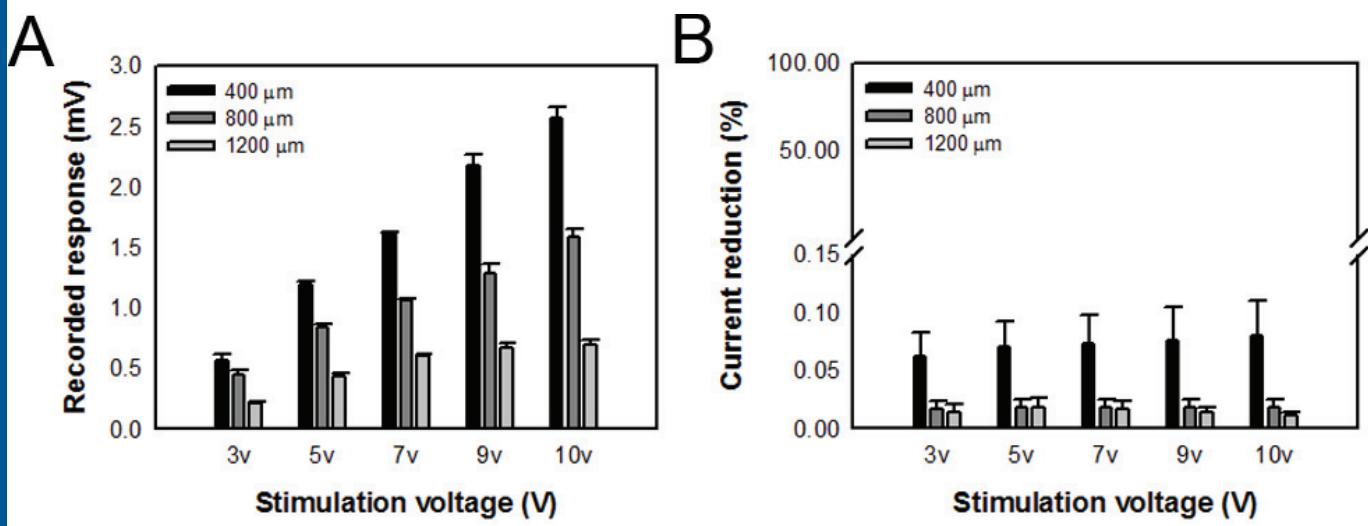
722

723 **Figure 16.** Co-expression of body pattern components in living oval squid. Various
724 body patterns of oval squids were observed in the wild and in the laboratory. These
725 body patterns are composed of different numbers of components. (A) Six body pattern
726 components are shown, though some of them were only weakly expressed. (B) Four
727 distinct components are expressed in this oval squid. (C) Three components are
728 shown in this male oval squid when attempting to mate with a female. (D) Four
729 components are differentially expressed in this squid. The numbers on the top indicate
730 the expression level of individual components.

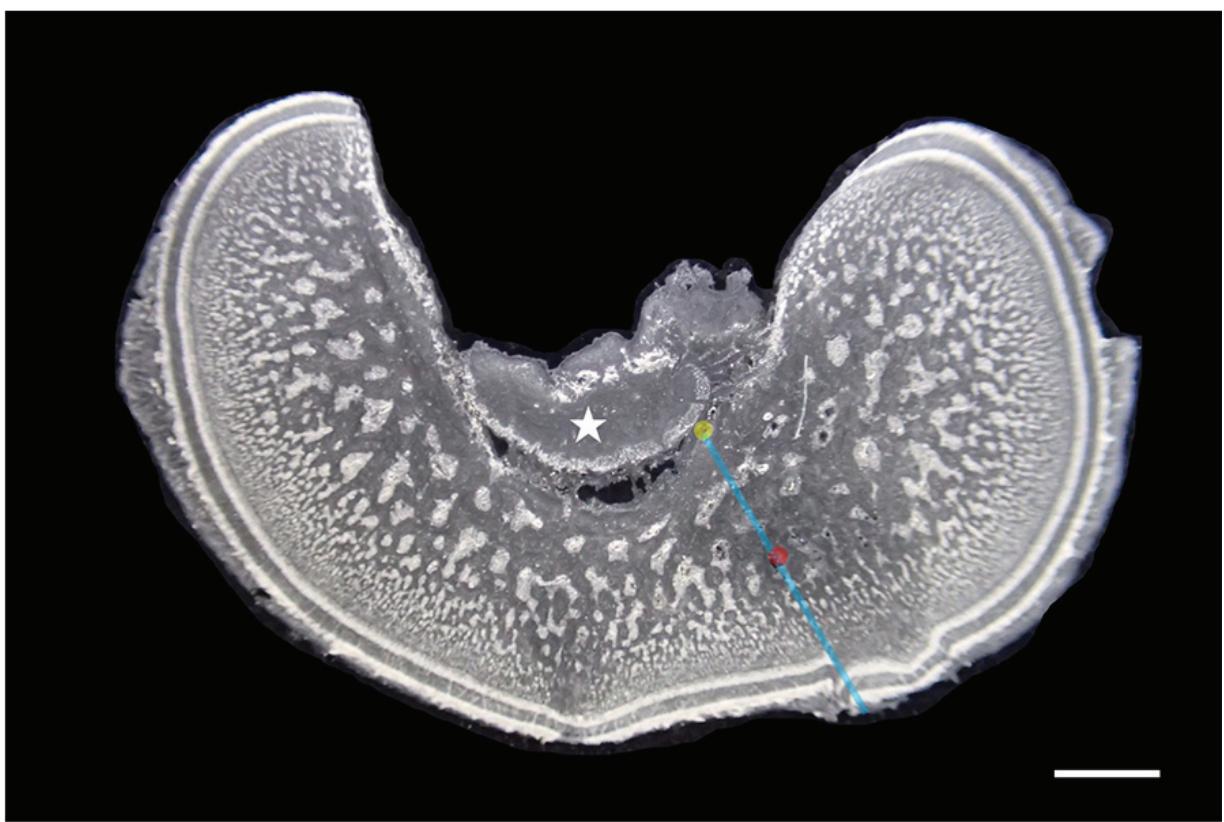
731

732 **Figure 17.** The mosaic organization of body pattern control in the optic lobe and the
733 downstream neural processing stages. (A) A conceptual diagram based on the findings
734 of the present study illustrates that the control units of individual body pattern
735 components are organized in a mosaic fashion in the motor command module
736 involved in body pattern generation. Each module contains all the control units of
737 body pattern components with different proportions. These modules are spread widely
738 across the medulla of the optic lobe. Thus, when stimulating any module in the optic
739 lobe, various numbers of different components can be evoked. In turn, different body
740 patterns can be generated by activating distinct sub-regions in the module. Note the
741 module depicted here represents a continuous structure of cell islands in the medulla.
742 (B) A flow chart shows the neural processing stages responsible for body pattern
743 generation. Visual information from eye are integrated by mosaic modules in the optic
744 lobe, then the optic lobe sends motor commands to the downstream lobes such as the
745 peduncle lobe, lateral basal lobe, and chromatophore lobe to control the
746 chromatophore organs.





A



B

