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Cultivation and Characterization of Planarian Neuronal Cells Isolated by Fluorescence Activated Cell Sorting (FACS)

Maki Asami[1,3], Tsuyoshi Nakatsuka[2], Tetsutaro Hayashi[3], Kenji Kou[4], Hiroaki Kagawa[4] and Kiyokazu Agata[3]*

[1]*Department of Biofunctional Chemistry, Faculty of Biomolecular Science, Okayama University Graduate School of Natural Science and Technology, 3-1-1 Tsushimanaka, Okayama 700-8535, Japan*
[2]*Department of Life Science, Faculty of Science, Himeji Institute of Technology, Harima Science Garden City, Hyogo, 678-1297, Japan*
[3]*Evolutionary Regeneration Biology Group, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan*
[4]*Department of Biology, Faculty of Science, University of Okayama, 3-1-1 Tsushimanaka, Okayama 700-8535, Japan*

ABSTRACT—Studies using molecular markers have revealed that planarians possess a highly organized brain. Here we separated brain neurons from dissociated planarian head cells by fluorescence activated cell sorting (FACS), and characterized them by single cell PCR analysis and cell culture. Dissociated cells were labeled with three different fluorescent dyes, Hoechst 33258, Merocyanine 540, and Propidium Iodide (PI), and fractioned by FACS. Interestingly, we have succeeded in identifying a cell fraction specific to the head, which we have named the head-abundant cell fraction (HAC). Most of the HAC expressed neuron-specific genes and proteins. When they were cultured *in vitro*, they showed an ability to extend neurites on several types of extracellular matrices (ECMs), and, depending on the ECM type used, presented a high level of plasticity in morphology and gene expression.

Key words: planarian, neuron, FACS, extracellular matrix, plasticity.

INTRODUCTION

The planarian CNS is composed of two morphologically distinct structures, an inverted U-shaped brain, and a pair of longitudinal ventral nerve cords (VNC) (Agata *et al*., 1998). It has been shown that several morphologically distinct domains are present in the brain, defined by differential expression of three homeobox genes, *DjotxA*, *DjotxB* and *Djotp* (Umesono *et al*., 1997; 1999). Recently, the molecular composition of the planarian brain has been extensively studied by expressed sequence tag (EST) and DNA chip analyses (Cebrià *et al*., 2002a–c; Mineta *et al*., unpublished). However, a physiological analysis has not been attempted as nobody has yet succeeded in culturing neuronal cells from planarians.

Planarian cells can be easily dissociated using their own proteases, secreted from the intestine. However, these proteases are toxic to the dissociated cells, including the neurons. Here we have introduced a fluorescence-activated cell sorting (FACS), to eliminate intestinal and dead cells from the dissociated cell mixture. By the simultaneous use of several fluorescence dyes, we collected a fraction that contained a high amount of neurons. We describe here how we obtained purified neuronal cells with FACS, and demonstrated subsequent gene expression. Finally, we succeeded in culturing the neuronal cells *in vitro*, and showed extracellular matrix (ECM)-type-dependent flexibility in differentiation of the cells.

* Corresponding author: Tel. +81-78-306-3085;
FAX. +81-78-306-3085.
E-mail: agata@cdb.riken.go.jp

MATERIALS AND METHODS

Animals

A clonal strain of the planarian *Dugesia japonica* (SSP) was used in this study. Individuals of approximately 8 mm in body length were selectively employed for cell sorting. To avoid contamination by debris from the gut contents, these animals were starved for more than one week before the experiment.

Dissociation of planarians into cells

Planarians were cut into three to four fragments on ice with a scalpel. Head fragments were collected from 50 animals and body fragments from 12 animals. To dissociate the cells, the fragments were soaked in Holtfreter's solution diluted 5/8 in distilled water (5/

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8 Holtfreter) containing 30 μg/ml trypsin inhibitor (TRYPSIN INHIBITOR Type II-O: Chicken Egg White: Sigma, St Louis, MO # T-9253), for several minutes. This treatment was essential for preparation of dissociated cells, by protecting them against the endogenous proteases. The fragments were then cut into smaller pieces, which were treated with 0.25% trypsin (Bact^{TM} Trypsin 250: DIFCO # 215230) solution for 1hr at 20°C. These samples were completely dissociated into single cells by gentle pipetting. To avoid the contamination of aggregated cells, the dissociated cell mixture was filtered using 40 μm pore size-filter (FALCON cell strainer: Becton Dickinson Labware # 352235).

Preparation for FACS analyses

The dissociated cells were stained with several fluorescent dyes: Propidium Iodide from Dojindo (Maryland, U.S.A.) (PI: excitation 536 nm; emission 617 nm, final concentration 0.54 μg/ml), Hoechst 33258 (Sigma: excitation 350 nm; emission, 450 nm, final concentration 0.89 μg/ml) and Merocyanine 540 (Sigma: excitation 555 nm; emission 578 nm, final concentration 0.92 μg/ml: Dragsten and Watt 1978), and incubated at 20°C for 20 min. The cells were

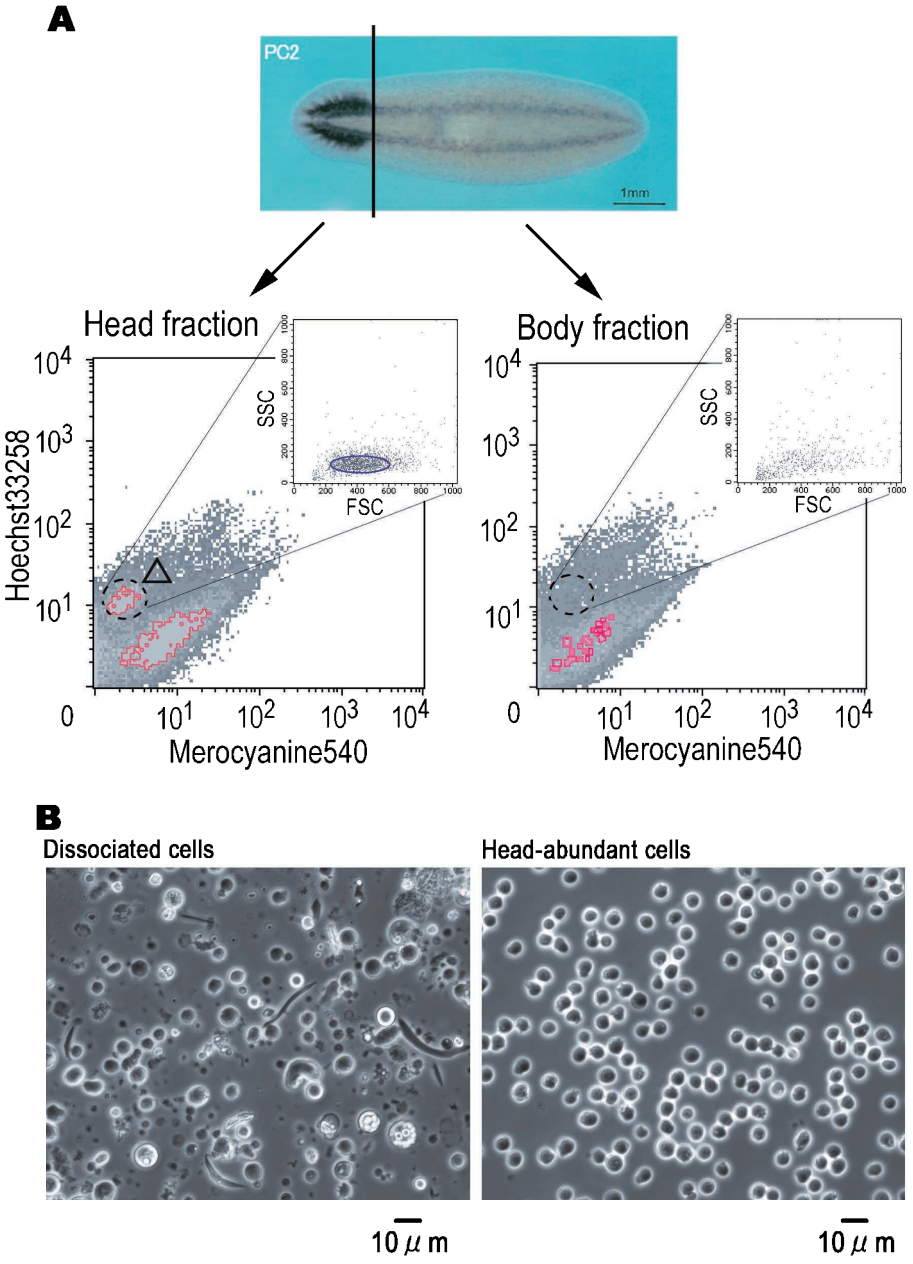


Fig. 1. Sorting of the head-abundant cell fraction (HAC) by FACS. A: *in situ* hybridization of *DjPC2* on a whole mount planarian, in which a brain and a pair of ventral nerve cords are stained. The lower panel shows typical dot-plot illustrations of dissociated cells from head (left) and body parts (right). Around 30% of total cells from each part were eluted as PI-positive cells before this analysis. The x-axis represents the relative fluorescence intensity of Merocyanine 540, and the y-axis represents the relative fluorescence intensity of Hoechst 33258. Red lines surround cell-abundant regions. An abundant dot-region specifically found in the head profile is indicated with the dotted circle. Profiles of cells from the circled regions based on side-angle light scatter (SSC) and forward-angle light scatter (FSC) are indicated at each right shoulder. B: Morphology of planarian cells before and after sorting. The dissociated planarian cells were composed of numerous types of cells including debris (left). The sorted cells fraction represents a homogeneous group of spherical cells 6–8 μm in diameter (right). Bar=10 μm

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then pelleted by centrifugation at 2,300 g for 2 min, and re-suspended at appropriate cell concentrations in 5/8 Holtfreter's solution. Flow cytometric analysis was performed using a FACS Vantage SE triple-laser flow cytometer (Becton-Dickinson). The dead cells were eliminated based on their staining intensity with the vital dye PI. Subsequent analyses were scored according to the cells' staining intensities with Hoechst 33258 for nuclear DNA and Merocyanine 540, as an indicator of membrane potential.

Culture of neuronal cells

The cells collected by FACS (2×10^4/dish) were cultured with MTTP medium (modified TTP medium; Teshirogi and Tohya, 1998) on several types of extracellular matrices (ECMs: Becton-Dickinson Falcon Biocoat Cellware: Laminin, catalog # 354458; Fibronectin, # 354457 and Poly-L-Lysine, # 554518).

For clear observation, the putative neuronal cells were cultured on glass-based culture dishes (MatTek Corporation, Ashland, MA: p35GC-0-10-C-gm) coated fibronectin (100 μg/cm^2) or laminin (60 μg/cm^2). The sorted cells were incubated for 30 min to layer onto the dishes, followed by replacement of the MTTP medium. The cells were cultured in a humidified incubator at 20°C.

Molecular characterization of planarian cells

Cell types of the sorted fractions were characterized as follows: From each cell culture, a cDNA template was constructed by reverse transcription polymerase chain reaction (RT-PCR) methods. This was further analyzed by the Gene Scan method (ABI: 3100 Genetic analyzer) with planarian-specific primers. The primers were specific as follows:

- *DjPC2* (forward primer: GGTGAAAATCCTCGTGGAAAATG; reverse primer: TATGGTCTATTTTGTGTTCCGTG; Agata *et al*., 1998);
- *Djsyt* (forward primer: GAACTCCGGCATTGGTCGGA; reverse primer: GTTAAGCTTTTGTTCATTAATTGTTTTC; Tazaki *et al*., 1999);
- *DjMHC-A* (forward primer: ATCGTAGTTCTGTGTCTACT; reverse primer: TTTTAAGCTTATCCTTCTCGT)
- *DjMHC-B* (forward primer: TCTAGATCTGTAAACCGTGGT; reverse primer: AATGTTTTCGAAGCTTTCTTT; Kobayashi *et al*., 1998)
- and *DjvlgA* (forward primer: CGCGCGCTCAGCAAGAATTT CCTACGA; reverse primer: GTGTGATCAGTCAGCGTC-CCACCA; Shibata *et al*., 1999).

Other neuronal-specific primers are listed in Table.2 (Kou *et al*., unpublished observations).

Immunocytochemical analysis

Polyclonal antibodies against the planarian α-synaptotagmin (DjSYT: Tazaki *et al*., 1999) were used to identify the planarian neurons. Immunocytochemical analysis was carried out as follows: The dissociated cells or cultured neuronal cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered-saline (PBS) for 1 hr at 4°C. Fixed samples were washed twice with 0.1% Triton X-100 in PBS (TPBS) for 5 min each at room temperature (RT), followed by blocking with 10% fetal bovine serum (FBS) in PBS for 30 min at RT. Samples were then incubated with the DjSYT antibody

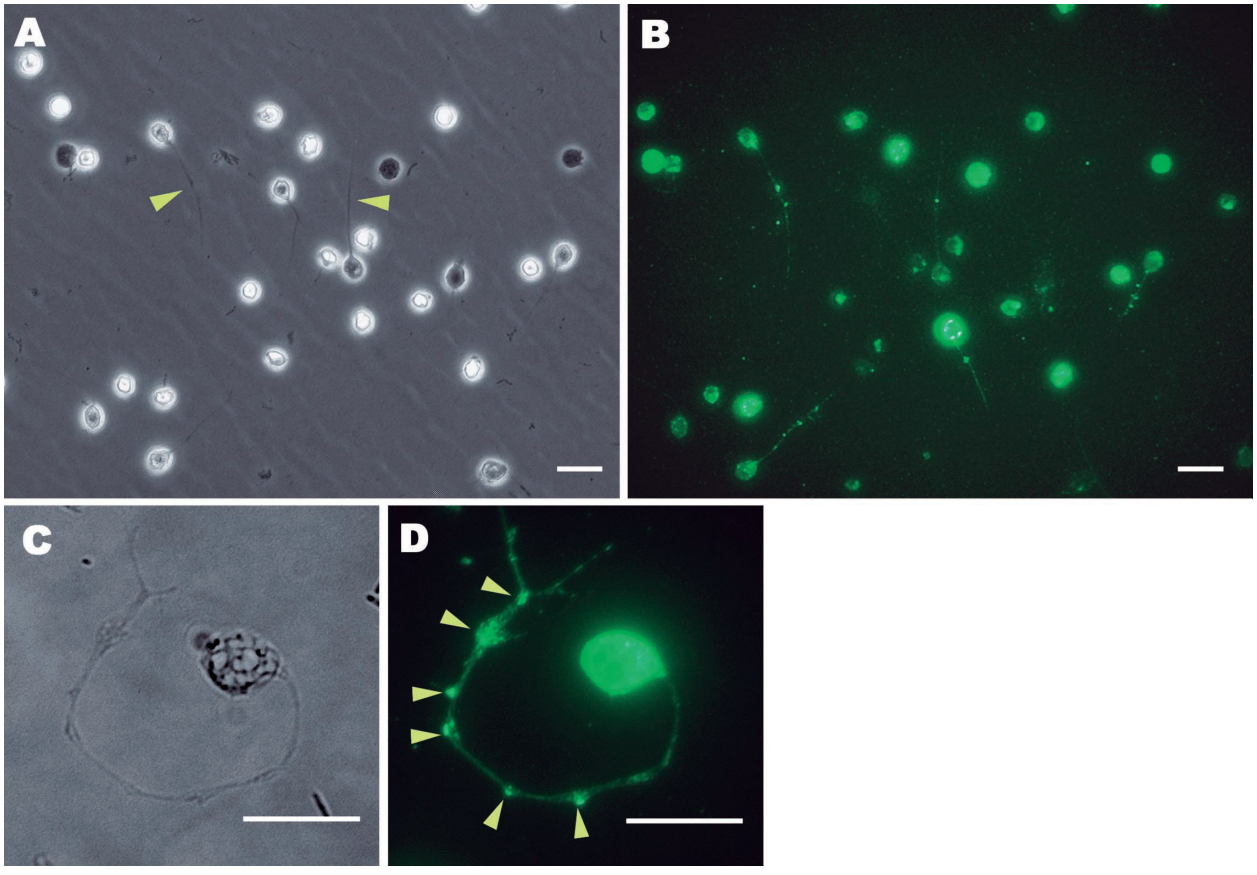


Fig. 2. Morphology and Immunocytochemistry of cultured head-abundant cells (HAC). A: A phase-contrast view of the cultured HAC. HAC cells were cultured on poly-L-lysine coated dishes for three days. The axons are indicated with arrowheads. B: Immunofluorescence image of cells stained with the anti-DjSYT antibody of (A). C: Higher magnification view of axonal HAC. D: Higher magnification view of the immunofluorescence image stained with anti-DjSYT antibody of (C). The varicosities are indicated with arrowhead. Bar=10 μm

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(diluted 1: 2000) for overnight on ice. After several washes with TPBS, the samples were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (diluted 1: 400; Molecular Probes) for 2 hr at RT. After washing with TPBS three times for 5 min each at RT, the samples were observed under a fluorescence microscope.

RESULTS

Identification of the head-abundant cell (HAC) fraction by FACS

Since planarians have a well-developed brain in the head (Agata *et al.*, 1998; Umesono *et al.*, 1999, 1997), a large amount of neuronal cells would be expected from this region (Fig. 1A). Thus, we prepared dissociated cells either from the head or the remaining body parts, and then compared the sorting profile on FACS to detect a head-specific cell fraction. To enhance the yield and purity of the neuronal cells, we used several different fluorescent dyes. PI vital staining was performed to exclude dead cells, which compromised approximately 30% of the total. The degree of forward-angle light scatter (FSC), which correlates with cell size, was also used to exclude non-cellular debris and numerous granule cells. The remaining cells, after the elimination of dead cells and debris, were analyzed based on the intensity of MC 540 staining (Dragsten and Watt, 1978) and of Hoechst 33258. The head-derived cells contained a fraction that could be characterized by weak staining with Hoechst 33258 and MC 540. This fraction was significantly abundant in the profile of the head as compared with the one of the body parts (Fig. 1A). The cells in this fraction were rather homogeneous in terms of their size and shape: round cells of 6–8 μm in diameter, less than 200 score in side-angle light scatter (SSC), and 200–600 score in FSC (Fig. 1B, right panel). These cells were not encountered among those derived from the other parts of the body. Thus, it seemed likely that these are highly specific to the head. We named this fraction the head-abundant cell fraction (HAC).

Characterization of HAC by cell culture and antibody staining

To characterize HAC, we performed cell culture. Some of the cultured cells showed extension of neurites on a poly-D/L-lysine-coated dish, gradually, after seeding (Fig. 2A, yellow arrows), and were positively stained with the anti-DjSYT

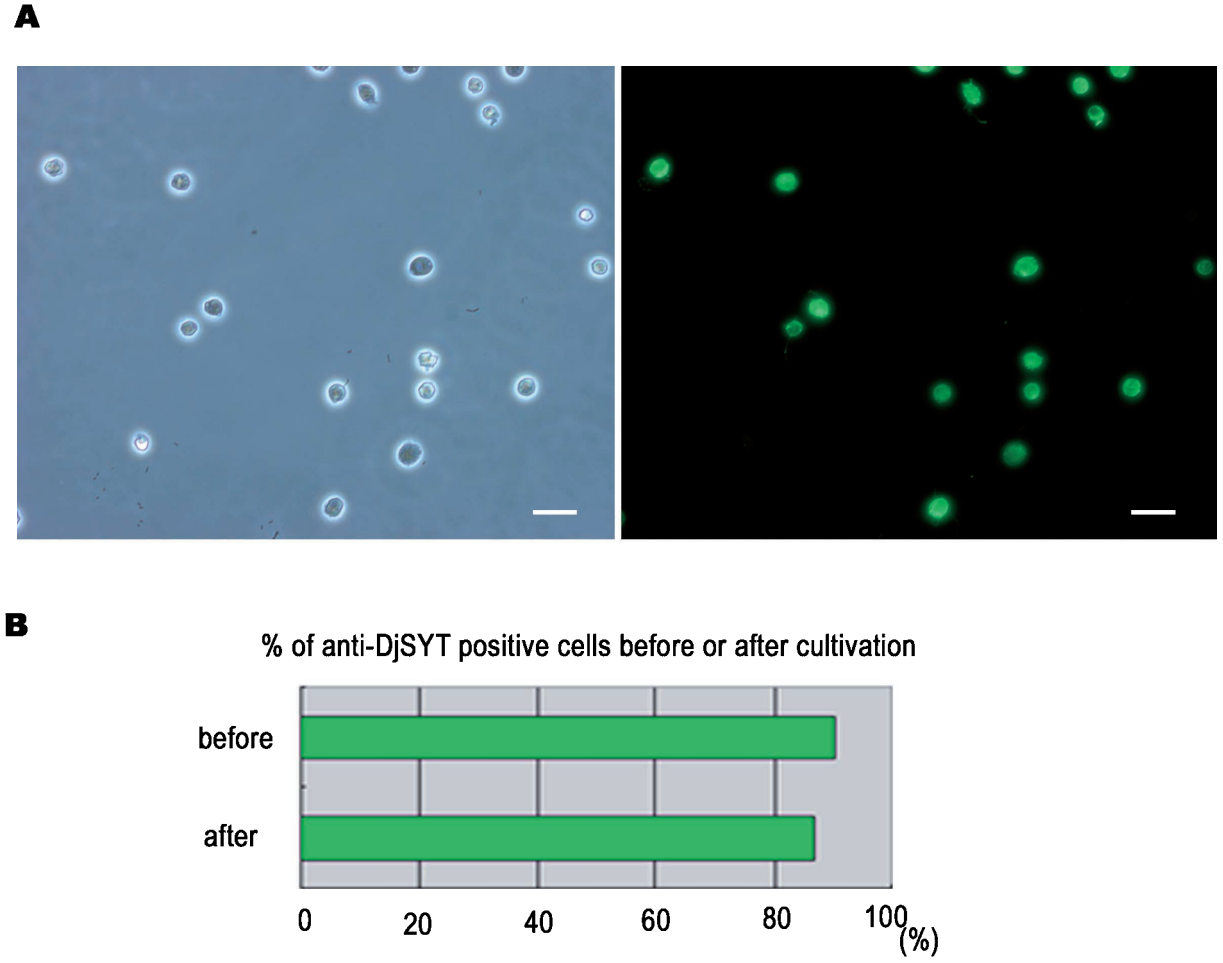


Fig. 3. Morphology and immunocytochemistry of HAC before cultivation. A: Fixed HAC cells just after sorting (left). The fixed HAC cells were stained with anti-DjSYT antibody (right). Bar=10 μm B: Summary of the percentage of DjSYT-positive cells before and after cultivation.

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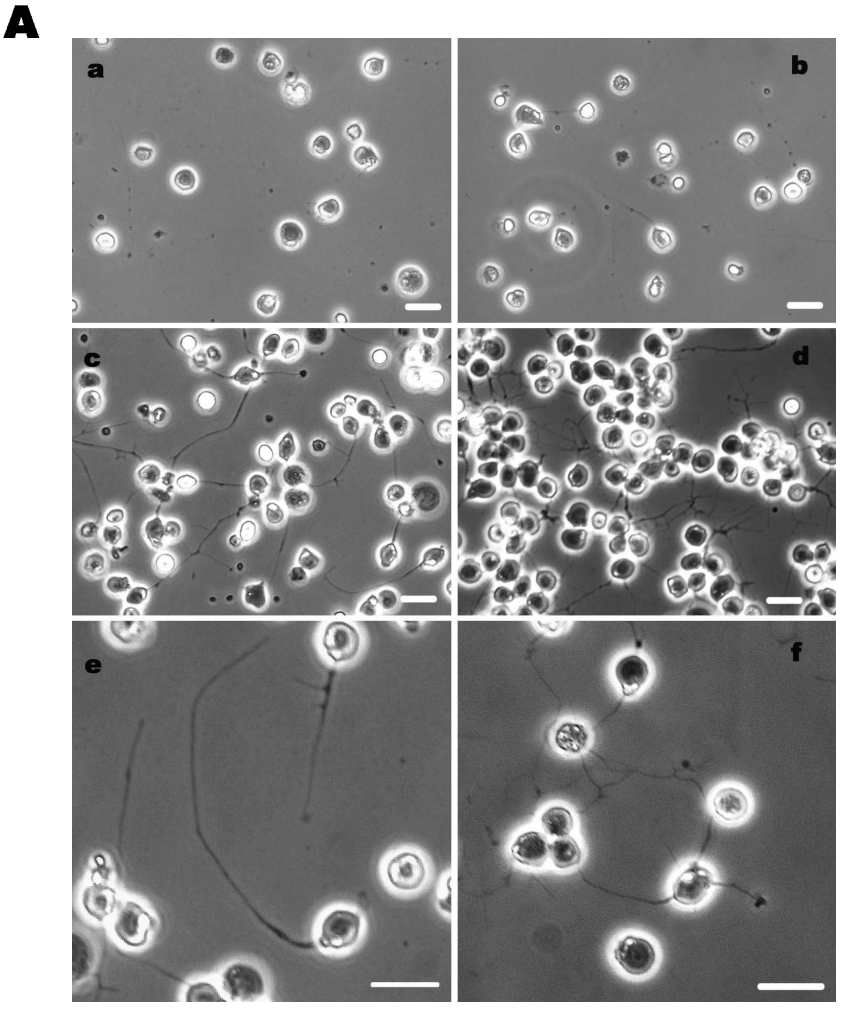
Table 1. A list of molecular markers used in this work.

Clone	Putative homologue	Primer FW (5'–3')	Primer RV (5'–3')
172_HH	AMPA-selective receptor subunit	AGTTTATAAATGCGCTCTTGAGTCTAGGAG	CAAACATCACTCTGGATTCGCCATTT
1008_HH	glutamate receptor (*DjGluR1*)	TGCTAACGCAGTTGATCATC	GGCGTGCAAACAGTTTGACA
4073_HH	N-methyl-D-asparate receptor	GAAAATTCGTTGCTGCTTCAAACCC	CGTATCACAGAACAAGTTTGCATTACC
776_HH	glutamate receptor	TAGACAACCTCTTTCTGGTATTGATAGCC	GGAATACTGGCTAATGTATCATGTGAACTG
4854_HH	GABA(A)receptor-associated protein	TGCTTTATATGAAGAGCATCCCGAA	GGTAAATGATTTAACTCCCCTACACGA
4307_HH	putative nicotinic acetylcholine receptor alpha 7-1 subunit	GCAAGAGTAATGGATAGATTCTGCTTAGTC	GGCGAAACTATGACTCTACTGGCAA
4808_HH	acetylcholine receptor protein alpha-5 chain receptor	CGTGGTACTAGTTCAATAGAGA	CAATCTATCCAACACCAAGC
6727_HH	purinergic receptor P2X	ACGGAGGAAAATTTGATCCTGCTACTTG	CCAATGGCTCATCTTTGGAATTACTCGG
4656_HH	Dopamine receptor	CGAATTGGCGATCGACTTAAATCTGCAC	TCCTATAATCGGGATTTTGTGAGCTTTCCA
1791_HH	G-protein alpha-subunit	CTGGAGCAAATAATTACGAAGAAGCCAGT	GTGTCAGTTGCGCAAGTGAAATGAGTG
1977_HH	guanine nucleotide binding protein beta 4	GTATGGGCAGCAAGAAAACGAAGCA	AAACAGGTCATCGATGGTTCTCGCTAA

Table 2. Molecular Characteristics of HAC before and after cultivation. HAC (indicated in Fig. 1A with blue-circle) and none-HAC (indicated in Fig. 1A with triangle) were characterized basing on RT-PCR wih cell-type specific primers. cDNA templates were prepared from 2×10^4 cells from each region or cells after three-days cultivated on different ECMs. The abbreviations were shown in Table 1. cDNA templates derived from whole intact planarian was used as positive-control on this analysis. Two individual experiments were performed, as a result, higher signal rather than each of the positive control on the scan analysis was indicated with (+), while lowere or undetectable one was indicated with (–).

Cell type specific markers		Before cultivation		After cultivation			
		HAC	**none-HAC**	**None**	**Poly D-lysin**	**FN**	**LN**
general	*DjEF1*	+/+	+	+/+	+/+	+/+	+/+
stem	*DjvlgA*	+/–	+	+/+	+/+	+/–	+/+
	DjPCNA	–/+	+	–/–	–/–	–/–	–/–
muscle	*DjMHC-A*	–/–	+	+/–	+/–	+/–	–/–
	DjMHC-B	–/+	+	+/+	+/–	–/–	+/+
neuron	*Djsyt*	+/+	–	+/+	+/+	+/+	+/+
	DjPC2	+/+	–	+/+	+/+	+/+	+/+
	DjPTP	+/+	+	+/+	+/+	+/+	+/+
	DjNCAM	+/+	–	+/+	+/+	+/+	+/+
	DjotxA	+/+	–	–/+	–/–	–/–	–/–
	DjotxB	+/–	–	+/+	+/–	+/–	–/+
	Djotp	+/+	–	–/+	–/+	–/–	+/+
	172_HH	–/+	–	+/+	+/–	–/–	+/+
	1008_HH	+/+	–	+/+	+/+	+/+	+/+
	4073_HH	+/+	–	+/+	+/–	+/+	–/–
	776_HH	+/–	+	+/+	–/–	+/+	+/–
	4854_HH	–/+	–	–/+	+/–	+/+	+/+
	4307_HH	–/+	–	+/+	+/+	+/–	+/+
	4808_HH	+/+	+	+/+	+/+	+/–	+/–
	6727_HH	–/–	+	+/–	–/–	+/–	–/+
	4656_HH	+/+	–	+/+	+/+	+/–	+/+
	1791_HH	+/+	+	+/+	+/+	+/+	+/+
	1997_HH	–/+	–	–/–	+/–	+/–	+/+

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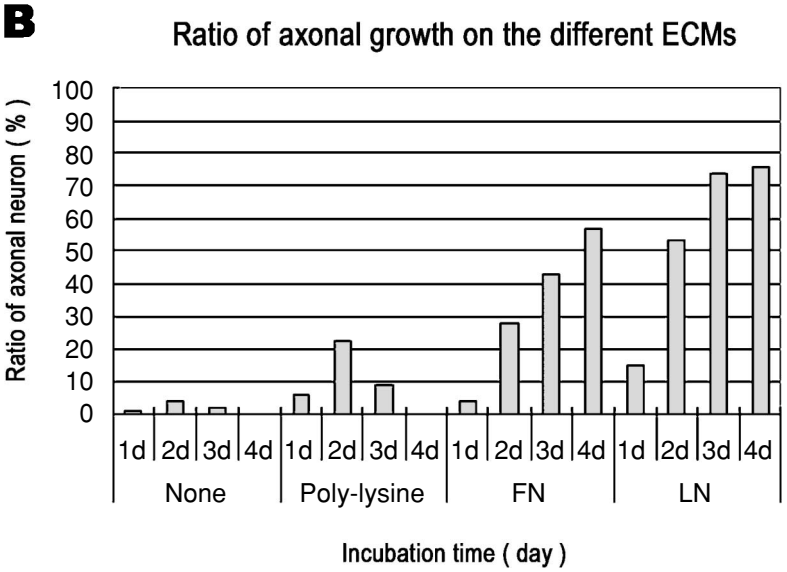


Fig. 4. Cultured HAC cells on different ECMs. A: Three day cultured HAC cells on the dishes (a) non-coated, (b) poly-D/L-lysine-coated, (c) fibronectin-coated and (d) laminin-coated. (e) and (f): Higher magnification views of axonal cells of c and d, respectively. Bar=10 μm B: The ratio of axon-growing cells on the different ECMs. Aliquots of 2×10^4 cells produced by FACS were cultured on the different substrate for four days. FN: fibronectin and LN: laminin. Numbers of the adhered cells and axon extending cells were counted, and each ratio of axon-extending neurons was calculated.

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anti-body (Fig. 2B). This neurite extension was observed from about 1 hr to 2 days of culture, and they tended to regress thereafter. When viewed at a higher magnification, several varicosities were observed (Figs. 2C, D). Furthermore, axonal flow was also observed under the microscope (data not shown). Additionally, about 90% of the collected cells showed intense immunoreactivity with anti-DjSYT both before and after culture (Figs. 3A, B). Thus, the HAC cells probably represented neurons that had differentiated at the time of sorting.

Characterization of HAC by molecular probes

To further characterize HAC cells, we analyzed gene expression by RT-PCR using cell-type specific gene primers including several neuronal probes. Table 1 shows a list of the molecular markers used in this analyses: 172_HH, 1008_HH, 4073_HH, 776_HH, and 4854_HH clones were used to detect amino acidergic neurons. Clone 1008_HH has been isolated as ionotropic glutamate receptor and named *DjGluR1* (Cebrià *et al*., 2002b). 4307_HH and 4808_HH clones were for cholinergic neurons, 4656_HH clone was for catecholaminergic neurons, 6727_HH clone was for purinergic neurons, and 1791_HH and 1997_HH clones were for G-protein coupled receptors. Clone 1791_HH specifically expressed in the brain branches, which is a marker for chemosensory neurons (Cebrià *et al*., 2002b). As shown in Table 2, HAC strongly expressed pan-neural markers such as *Djsyt*, *DjPC2* and *DjPTP*, and brain specific markers such as, *DjotxB* and *Djotp*. The other neural-specific genes, except for the purinergic receptor P2X, were also uniformly detected in HAC cells. On the other hand, expression of these genes was barely detected in a control fraction (Fig. 1A, marked by a triangle). These results suggest that HAC contains a variety of brain-associated neuronal cells.

Culture of HAC fraction on different ECMs

To extend the viability of the HAC *in vitro*, the effects of several types of ECMs including fibronectin, and laminin, were tested. Two or 4×10^4 cells of the HAC were inoculated on each coated dish. The cultured HAC developed elongated neurites. The peak of extension was observed at several days after cultivation, with various rates of extension depending on the types of ECM (Fig. 4B). When they were cultivated on ECMs, stable adhesion and prolongation of viability were observed as compared with the cultures on non-coated, or poly-D/L-lysine-coated dishes (See Fig. 4B). Fibronectin and laminin had particularly conspicuous affects on neurite extension (Figs. 4Ac, Ad). The cultured HAC cells also showed various morphologies depending on the type of ECM. Cells cultured on fibronectin tended to develop neurites in a uni-polar manner, whereas those cultured on laminin showed bi-polar or multi-polar morphology (compare Figs. 4Ae and Af). The expression patterns of neural genes in the cells cultured on several different ECMs were analyzed. As shown in Table 2, all of the samples highly expressed planarian pan-neural markers including *Djsyt*, *DjPC2*, *DjPTP* and *DjNCAM*. However, the cells cultured on fibronectin did not express *Djotp* or 172_HH (AMPA type Glutamate receptor), which were strongly expressed in the cells cultured on laminin. By contrast, expression of 4073_HH (NMDA type Glutamate receptor) was suppressed in the cells cultured on laminin. *DjotxA* was not detected in the cells cultured with ECMs, while expressions of *DjotxB* and *Djotp* were generally maintained. These results indicate that the isolated brain derived-neuronal cells show high levels of plasticity for gene expression as well as varied morphology, depending on the different ECMs used.

DISCUSSION

Purification of neurons by FACS

At the initial stage of this study, we did not expect that FACS would allow us to purify neurons, as we had no cell surface antibody specific against planarian neurons. We expected that FACS might be a powerful tool to eliminate toxic cells after dissociation. However, we found that we could identify the neural cell-populations by comparing the sorting profile between head and body regions. We successfully isolated and cultured planarian brain neurons with 80–90% purity (Fig. 1). This suggests that FACS can be used to purify a certain population of cells by the simple combination of fluorescence dyes, even though specific antibodies are not available.

In this study, we applied a cell membrane potential-sensitive dye, MC 540, which is used to detect neuronal cells in both vertebrates and invertebrates (Ross *et al*., 1977). Unexpectedly, HAC was obtained from the MC 540 weakly-positive region, not from the strongly stained region (see Fig. 1A), which is inconsistent with the physical features of MC 540 (Cohen *et al*., 1974; Tasaki *et al*., 1973). We speculate that the HAC cells might have lost their axons, the targets of MC 540, after dissociation procedures (Fig. 2A). Unexpectedly, neurites of the HAC begin to be regenerated during culture (Figs. 2B and 3). In fact, based on the observation of neurites in planarian brain with the DiI tracing technique, the average length of the neurites of the brain neurons are more than 100 μm *in vivo* (data not shown).

We have thus succeeded in establishing a cell culture system for planarian neurons that, for example, will potentially provide us with a tool for further electrophysiological analysis. In preliminary experiments, it has been found that the HAC cells show a firing response to glutamate stimulation (Oka, personal communication).

The morphological plasticity and molecular characterization of planarian neuronal cells

To prolong the viability of cultured neuronal cells, we inoculated the cells on different ECMs, fibronectin and laminin. Through investigations on vertebrates and invertebrates, it has been well known that ECM molecules play important roles in neuronal cell migration, axonal extension

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and cell survival (Lochter *et al*., 1995; McAllister 2000; Fitzakerley 2001). Laminin and fibronectin support growth cones the most prominently among several classes of ECM substrates (Lochter *et al*., 1995). We found that the viability of HAC and neurite survival also significantly increased in the presence of laminin or fibronectin (Fig. 4). These results suggested that the ECM molecules might have similar functions in planarian CNS neuronal cells as found in other animals. In the planarian body, it has been reported that the ECM forms a meshwork arrangement that apparently envelops the individual muscle cells to form a layer at the base of the epidermis (Rieger *et al*., 1991). Moreover, Lindroos (1990) also reported that fibronectin, laminin and collagen type-IV were immunohistochemically detected in ECMs of another Turbellarian, *Polycelis nigra* (Lindroos and Still, 1990). Interestingly, fibronectin and laminin showed different affects on neurite outgrowth and gene expression in planarians. To investigate whether the cells can really change the morphology of their neurites depending on the ECM, or whether different cell types are simply selected by different affinity of cell-to substrate, we counted cell numbers and monitored cell behavior (data not shown). These data indicated that most of the HAC cells can adhere to both fibronectin and laminin, but change their morphologies and gene expression, suggesting that their phenotype can be affected by different substrates. Still, we could not demonstrate actual functional changes at molecular levels. We need to clarify these functional differences by electrophysiological studies. The *in vitro* culture of planarian neuronal cells will prove a valuable step for future functional analyses to understand the planarian CNS.

Similar plasticity in neuronal cells has also been observed in Hydra (Koizumi *et al*., 1988; Zhang and Sarras, 1994). It is known that Hydra neurons change morphology and functions in the process of their continuous movement along the anterior-posterior axis. Although the migration of neuronal cells has not been described, it is possible to speculate that such migration events may also occur during CNS regeneration, growth or degrowth in the planarian CNS (Agata and Watanabe, 1999; Kato *et al*., 1999; Kobayashi *et al*., 1999; Orii *et al*., 1999).

Here we have shown that FACS is a powerful tool for the isolation of specific cell types from planarians, even though we did not use any specific cell surface antibodies. We recently found that some specific cell populations are eliminated by X-ray-irradiation. We expect that such approaches using FACS will enable us to isolate stem cell populations from dissociated planarian cells in the near future.

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