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A Primary Culture System for Functional Analysis of *C. elegans* Neurons and Muscle Cells

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Summary

***C. elegans* has provided important insights into neuromuscular system function and development. However, the animal's small size limits access to individual neurons and muscle cells for physiological, biochemical, and molecular study. We describe here primary culture methods that allow *C. elegans* embryonic cells to differentiate into neurons and muscle cells in vitro. Morphological, electrophysiological, and GFP reporter studies demonstrate that the differentiation and functional properties of cultured cells are similar to those observed in vivo. Enriched populations of cells expressing specific GFP reporters can be generated by fluorescence-activated cell sorting. Addition of double-stranded RNA to the culture medium induces dramatic knockdown of targeted gene expression. Primary nematode cell culture provides a new foundation for a wide variety of experimental opportunities heretofore unavailable in the field.**

Introduction

The nematode *C. elegans* provides a powerful model system in which to explore the molecular basis of cellular differentiation and function. Completion of the nematode genomic sequence and development of RNA interference (RNA_i) (Fire et al., 1998) and gene knockout methods (Liu et al., 1999) have allowed the ablation of specific genes to establish their functions in vivo. *C. elegans* development is precisely defined, with a detailed record of the birth and differentiation of every cell that contributes to the adult animal (Sulston et al., 1983). Lastly, the morphology and connectivity of each of the 302 neurons in the *C. elegans* nervous system have been defined using serial section electron microscopy (White et al., 1986).

Despite these many experimental advantages, the small size of most somatic cells and the tough, pressurized cuticle surrounding the animal limit access for molecular studies of cell-specific gene expression and function. For example, individual cells or organs cannot be readily isolated in quantities sufficient for the generation of cDNA libraries and for detailed profiling of gene and protein expression patterns.

The small cell size and pressurized cuticle also limit access for detailed functional studies by electrophysiological and optical approaches. Elegant patch clamp methods have been developed to study ion channel activity in body wall muscles (Richmond et al., 1999; Richmond and Jorgensen, 1999) and in a few neuron types (Goodman et al., 1998; Lockery and Goodman, 1998; Pierce-Shimomura et al., 2001). However, these methods are technically demanding. Optical methods employing proteins such as GFP and Ca^{2+}-sensitive cameleons (Miyawaki et al., 1999) hold promise for monitoring excitable cell intracellular Ca^{2+} levels (Kerr et al., 2000) and membrane potential (Khatchatouriants et al., 2000) in vivo. Whole animal imaging studies, however, do not readily allow detailed mechanistic study of ion channel activity and its regulation.

A nematode primary cell culture system would provide direct access to individual cell types for functional and molecular analyses. Because of past technical hurdles, large-scale cell culture methods have not been widely exploited for the study of *C. elegans*. However, intact embryos or small numbers of blastomeres have been cultured and used to study *C. elegans* developmental events. For example, Edgar and coworkers developed methods to isolate and culture single nematode embryos that allow the study of cell division and morphogenesis (Edgar, 1995). Isolated E blastomeres differentiate into epithelial cells that produce apical tight junctions, express proteins exhibiting a polarized distribution, and surround an extracellular space analogous to the intestinal lumen (Leung et al., 1999).

Early attempts at large-scale culture of *C. elegans* embryonic cells have been described (Bloom, 1993). This pioneering work demonstrated the feasibility of culturing differentiated neurons from *C. elegans*. However, Bloom noted significant problems with cell survival, attachment of cells to the growth substrate, cell differentiation, and reproducibility of the methods. Initial attempts to patch clamp cultured cells were unsuccessful. Buechner et al. (1999) have also reported that cultured *C. elegans* embryonic cells undergo morphological differentiation resembling neurons and muscle cells.

We describe here methods that allow the robust, large-scale culture of *C. elegans* embryonic cells. Isolated embryonic cells differentiate into the various cell types that comprise the newly hatched L1 larva. Expression of a number of cell-specific GFP reporters and molecular markers is similar to that observed in the intact animal. Fluorescence-activated cell sorting (FACS) can be used to isolate GFP-marked cells in quantities sufficient for molecular studies. We demonstrate that body muscles and identified neuron types can be readily patch clamped in the conventional whole-cell mode. Whole-cell electrophysiological properties are similar to those measured in vivo. Finally, we show that addition of double-stranded RNA (dsRNA) to the culture medium induces knockdown of targeted gene expression in both neurons and muscle cells. Primary *C. ele-*

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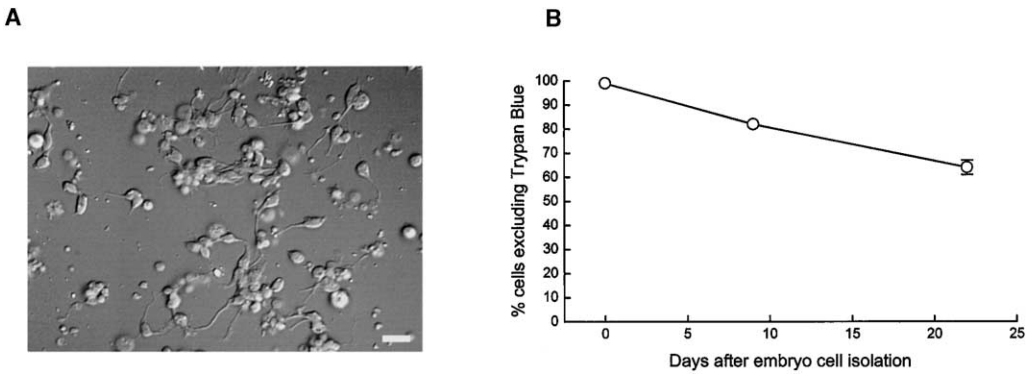

Figure 1. Morphology and Survival of Cultured *C. elegans* Embryonic Cells

(A) Differential interference contrast (DIC) image of a typical culture of *C. elegans* embryonic cells 4 days after isolation and plating. Scale bar is 5 μm.

(B) Trypan blue exclusion in freshly isolated embryonic cells (day 0) and cells cultured for 9 and 22 days.

gans cell culture offers new opportunities for defining complex excitable cell processes at the molecular level.

Results

Embryonic Cells Undergo Morphological Differentiation In Vitro

Freshly isolated embryonic cells are spherical in shape and vary in size from ~2 to 10 μm in diameter. Morphological differentiation is observed within 2–3 hr after cell isolation and plating. As cells begin to adhere to the growth substrate, many were observed to send out neuron-like processes. Extensive morphological differentiation was observed within 24 hr. The majority of cells in culture had a neuron- or muscle cell-like morphology (Figure 1A).

Cells must adhere to the growth substrate in order for morphological differentiation to occur. Morphological differentiation is extensive when cells are plated onto glass surfaces coated with peanut lectin (Figure 1A). A similar degree of differentiation was observed in cells cultured on substrates coated with poly-L-lysine or commercially available (Becton Dickinson Labware) glass coverslips coated with a mixture of laminin and poly-D-lysine. However, cells grown on substrates coated with laminin, fibronectin, or collagen IV did not adhere. Failure to adhere led to the formation of large cell clumps. Single cells present in these nonadherent cultures showed very limited morphological differentiation 24–72 hr after plating. Varying the concentration of these coating agents over a 10-fold range had no obvious effect on cell adhesion or differentiation.

Trypan blue staining demonstrated that >99% of embryonic cells survive the isolation procedure. At 9 and 22 days after plating, >85% and >65%, respectively, of the cells exclude trypan blue (Figure 1B). Cell survival appeared to be optimal using L-15 cell culture medium containing 10% fetal bovine serum. In the absence of serum, cells exhibited little morphological differentiation. Increasing medium osmolality to 340 mOsm by sucrose appeared to enhance both morphological differentiation and cell viability.

Cell-Specific GFP Reporters Are Expressed in Culture

A powerful experimental advantage of *C. elegans* is the relative ease and economy of generating transgenic animals. It is a mainstay in the field to use GFP reporters to determine cell types in which a particular gene is expressed (Chalfie et al., 1994). We exploited the availability of GFP transgenic lines to establish cultures in which specific cell types are marked by GFP expression.

Table 1 shows GFP reporter lines from which cells were cultured. After isolation, cells were seeded at a density of 340,000–375,000 cells/cm^2 in 8-well chambered coverglasses. Cultures were imaged by brightfield and fluorescence microscopy at defined intervals for 72 hr after plating. The frequency of GFP-expressing cells in the cultures was calculated as the percent of the total number of cells exhibiting well-formed processes, which we defined as neurons and muscle cells.

GFP-expressing cells prepared from an *unc-119*::GFP worm strain were abundant and easily detected in cultures examined 2–3 hr after isolation and plating. *unc-119* encodes a highly conserved protein of unknown function that is expressed in all *C. elegans* neurons and neuronal precursor cells (Maduro and Pilgrim, 1995; Maduro et al., 2000) and in eight body wall muscles located in the head (see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/33/4/503/DC1). Newly hatched L1 larvae have 81 body wall muscle cells and 222 neurons. Of these cells, 230 or 76% should express UNC-119. In culture, *unc-119*::GFP expression was detected in 74%–76% of all cells with neuron and muscle cell-like morphology (Table 1). Immunostaining experiments demonstrated that a subset (14%) of body wall muscle cells express *unc-119*::GFP in culture (see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/33/4/503/DC1). These values are in excellent agreement with the frequency of *unc-119*::GFP expression expected in neurons and muscle cells of newly hatched L1 larvae.

Fluorescence micrographs of various GFP-expressing cell types are shown in Figure 2. *myo-3* encodes a specific myosin heavy chain isoform that is expressed in all body muscles (Ardizzi and Epstein, 1987; Fire et al., 1998; Miller et al., 1983). *myo-3*::GFP-expressing muscle

Table 1. GFP Reporters Expressed in Cultured Cells

Gene	Encoded Protein	In Vivo Expression Pattern	GFP Expression after Cell Isolation	[1]Frequency of GFP-Expressing Cells in Cultures Observed 24–72 hr after Plating		
				24 hr	48 hr	72 hr
unc-119	Novel	All neurons and neuronal precursor cells; 8 head muscle cells	Yes; very abundant[3]	76%	74%	75%
unc-4	Transcription factor	13 embryonic motor neurons; 20 larval motor neurons	Yes; rare[3]	4.8%	5.4%	5.4%
mec-7	β-tubulin	4 embryonic touch neurons; 2 larval touch neurons	Yes; rare	5.4%	4.3%	5.8%
opt-3	H^+/oligopeptide transporter	2 embryonic glutamatergic neurons; also weakly expressed in 4–6 neurons not yet identified	Yes; rare	3.4%	2.9%	3.3%
myo-3	Myosin-3	Body muscles[2]	Yes; abundant[3]	31%	34%	30%

[1]Cultures were grown in 8-well chambered coverglasses. At various times after plating, 9–16 random fields were imaged at 40× by brightfield and fluorescence microscopy. The frequency of GFP-expressing cells in the cultures was calculated as the percent of the total number of cells exhibiting well-formed processes.
[2]Virtually all cells expressing neuron-specific GFP reporters had well-developed processes. However, processes were not evident on all *myo-3*::GFP-expressing cells. Only *myo-3*::GFP-positive cells with observable processes were included in the analysis of expression frequency.
[3]FACS analysis demonstrated that *unc-119*::GFP, *unc-4*::GFP, and *myo-3*::GFP were expressed in 87%, 0.2%, and 4%, respectively, of freshly isolated embryonic cells.

cells typically exhibited a spindle-shaped morphology with single elongated processes at both ends of the cell (Figure 2A). Similar processes are also seen in vivo as muscle cells extend "arms" to form synapses with motor neurons (White et al., 1986). Muscle cells in culture also occasionally exhibited a Y-shaped morphology with two processes emanating from a single pole of the cell. Figure 2F shows a Y-shaped muscle cell physically interacting with an *unc-4*::GFP-expressing neuron. *unc-4* encodes a homeodomain transcription factor (Miller et al., 1992) expressed in the 13 embryonic cholinergic motor neurons (Lickteig et al., 2001; Miller and Niemeyer, 1995). Motor neurons form neuromuscular junctions with striated body wall muscles in vivo (White et al., 1986). The interaction between muscle cells and motor neurons suggests the possibility that synapses may form between cultured cells.

mec-4 and *mec-7* encode a degenerin-type ion channel (Huang and Chalfie, 1994) and β-tubulin (Hamelin et al., 1992), respectively. Both genes are expressed largely in touch neurons (Hamelin et al., 1992; Huang and Chalfie, 1994). Four touch neurons (ALML/R and PLML/R) develop during embryogenesis, and two (AVM and PVM) arise during larval development. We cultured embryonic cells isolated from *mec-4*::GFP- and *mec-7*::GFP-expressing worms. *mec-7*::GFP expression was heterogeneous, ranging from barely detectable to intense. Both *mec-4*::GFP-expressing cells (Figure 2B) and cells expressing intense *mec-7*::GFP fluorescence (data not shown) had extensive processes that were highly ramified. These processes were considerably longer than those observed in other neuronal types (compare Figures 2B–2D).

The frequency of various GFP-expressing cell types observed after at least 24 hr in culture is similar to that observed in the intact, fully developed embryo (Table 1). For example, ~5% of all cells with well-developed processes express *unc-4*::GFP in culture, a finding consistent with the fact that *unc-4*::GFP is expressed in 13 embryonically derived motor neurons in vivo (Lickteig et al., 2001; Miller and Niemeyer, 1995).

Neurons and Muscles Express Cell-Specific Markers In Vitro

In addition to cell-specific GFP reporters, we also assayed for expression of other markers of cell differentiation. The synaptic vesicle protein, synaptotagmin (SNT-1), is expressed widely in the *C. elegans* nervous system (Nonet et al., 1993), including the *unc-4*::GFP-marked A-type motor neurons of the ventral nerve cord (Lickteig et al., 2001). As shown in Figure 2C, SNT-1 immunolocalizes to a single process extending from an *unc-4*::GFP neuron in culture. Other *unc-4::*GFP neurons also showed SNT-1 immunostaining in the soma (data not shown). In no case did we observe *unc-4*::GFP neurons that failed to express SNT-1.

The myosin heavy chain genes, *unc-54* and *myo-3*, are coexpressed in body wall muscle in vivo (Ardizzi and Epstein, 1987; Miller et al., 1983). As shown in Figure 2A, UNC-54 is also coexpressed with *myo-3*::GFP in cultured muscle cells. Conversely, of 389 *myo-3*::GFP cells examined, only one immunostained for the neuron-specific marker SNT-1 (see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/33/4/503/DC1).

The reporter genes *unc-4*::CFP and *acr-5*::YFP are expressed in A-type and B-type motor neurons in the ventral nerve cord, respectively (Figure 2G). *acr-5*::YFP is also detected in separate clusters of neurons in the head and tail that do not express *unc-4::*CFP (Miller et al., 1999; Winnier et al., 1999). We cultured cells from a worm strain expressing both *unc-4::*CFP and *acr-5*::YFP transgenes (Figure 2G). In over 300 cultured CFP- and 900 YFP-positive cells examined, none showed coexpression of the two markers, indicating that these reporter genes retain their neuron-specific expression patterns in vitro.

Postembryonically Derived Motor Neurons Are Not Observed in Culture

An important question is whether cells in culture undergo postembryonic differentiation. To test for this possibility, we cultured cells from a *del-1*::GFP transgenic

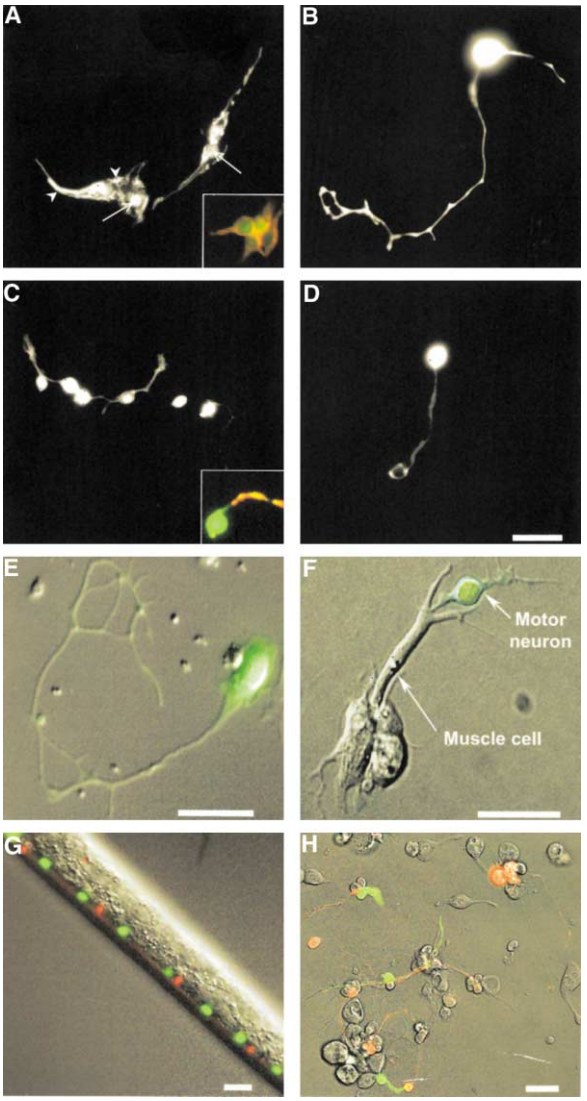

Figure 2. Micrographs of Cultured Embryonic Cells Expressing Cell-Specific GFP Reporters

(A) *myo-3*::GFP expression in cultured body muscle cells. *myo-3* encodes a myosin heavy chain isoform expressed in body muscles. The *myo-3* reporter strain expresses two GFPs with peptide signals that target them to either the nucleus (arrows) or mitochondria (arrowheads). Inset: immunolocalization of UNC-54 myosin in body wall muscle cells. Cultures derived from *myo-3*::GFP-expressing worms were fixed and incubated with anti-UNC-54 and a Cy3-conjugated (red) secondary antibody. Yellow indicates overlap of GFP and Cy3 fluorescence.

(B) Cultured mechanosensory neuron expressing *mec-4*::GFP. *mec-4* encodes a degenerin-type ion channel subunit expressed largely in neurons that respond to gentle body touch.

(C) Cultured cholinergic motor neurons expressing *unc-4*::GFP. *unc-4* encodes a homeodomain transcription factor. Inset: immunolocalization of synaptotagmin in motor neurons. Cultures derived from *unc-4*::GFP-expressing worms were fixed and incubated with anti-synaptotagmin and a Cy3-conjugated (red) secondary antibody. *unc-4*::GFP, anti-SNT-1, and DAPI fluorescence are shown in green, red, and blue, respectively. Yellow indicates overlap of GFP and Cy3 fluorescence.

(D) Cultured neuron expressing *opt-3*::GFP. OPT-3 is a H^+/oligopeptide transporter expressed in glutamatergic neurons.

(E) Combined DIC and fluorescence micrograph of an *unc-119*::GFP-expressing neuron. *unc-119* encodes a novel protein that is expressed in all neurons and eight head muscle cells.

(F) Combined DIC and fluorescence micrograph of an *unc-4*::GFP-expressing cholinergic motor neuron physically interacting with a body wall muscle cell.

(G) Lateral view of a transgenic larval worm showing expression of *unc-4*::CFP (green) in A-type motor neurons and *acr-5*::YFP (red) in B-type motor neurons in the ventral nerve cord. Anterior is to left.

(H) *unc-4*::CFP and *acr-5*::YFP are expressed in separate sets of neurons in vitro after 5 days in culture. All scale bars are 10 μm.

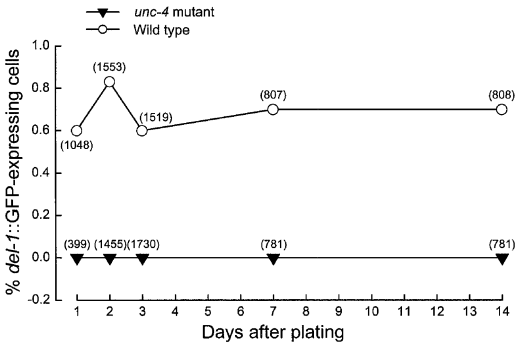

Figure 3. Expression of *del-1*::GFP in Wild-Type and *unc-4* Mutant Worms

del-1 encodes a DEG/ENaC-like channel that is expressed in two embryonic neurons and in 23 neurons in L2–L4 larvae and adults. In cultures derived from wild-type worms, GFP is detected in 0.6%–0.8% of cells exhibiting neuronal and muscle morphology. GFP expression is not detected in cultures prepared from *unc-4* mutant worms. Null mutations in *unc-4* prevent expression of *del-1* in embryonic neurons, but do not block expression in larvae and adults. Cultures were imaged by brightfield and fluorescence microscopy daily for 14 days after cell isolation and plating. Numbers in parentheses are the total number of cells observed with neuronal and muscle morphology.

line for 2 weeks. *del-1* encodes a DEG/ENaC-like channel (Tavernarakis and Driscoll, 1997) that is expressed in two embryonic neurons, SABVL and SABVR. In L2 and L3 larvae, *del-1* is also expressed in 11 VB neurons and 12 VA neurons (Winnier et al., 1999). Embryonic SABV neurons represent 0.66% of the total number of neurons and body wall muscles present in the newly hatched L1 larva. As shown in Figure 3, *del-1*::GFP expression was detected in vitro at a frequency of 0.6%–0.8%. This frequency is very similar to that observed in the fully differentiated embryo but much less than the expected number of *del-1*::GFP neurons if the postembryonic VA and VB motor neurons (~6% of 397 adult neurons and body wall muscles) were also appearing in culture.

del-1::GFP expression is eliminated from embryonic SABVL and SABVR in *unc-4* mutant worms but is retained in VA and VB motor neurons (Winnier et al., 1999) (D.M.M., unpublished data). We cultured cells from a *del-1*::GFP line in an *unc-4* mutant background. As shown in Figure 3, no *del-1*::GFP-positive cells were detected in vitro. This finding is also consistent with the conclusion that VA and VB motor neurons are not differentiating in culture. Taken together, the experiments shown in Figure 3 indicate that embryonic cells cultured in vitro may not undergo postembryonic development. However, it is conceivable that other gene expression patterns may

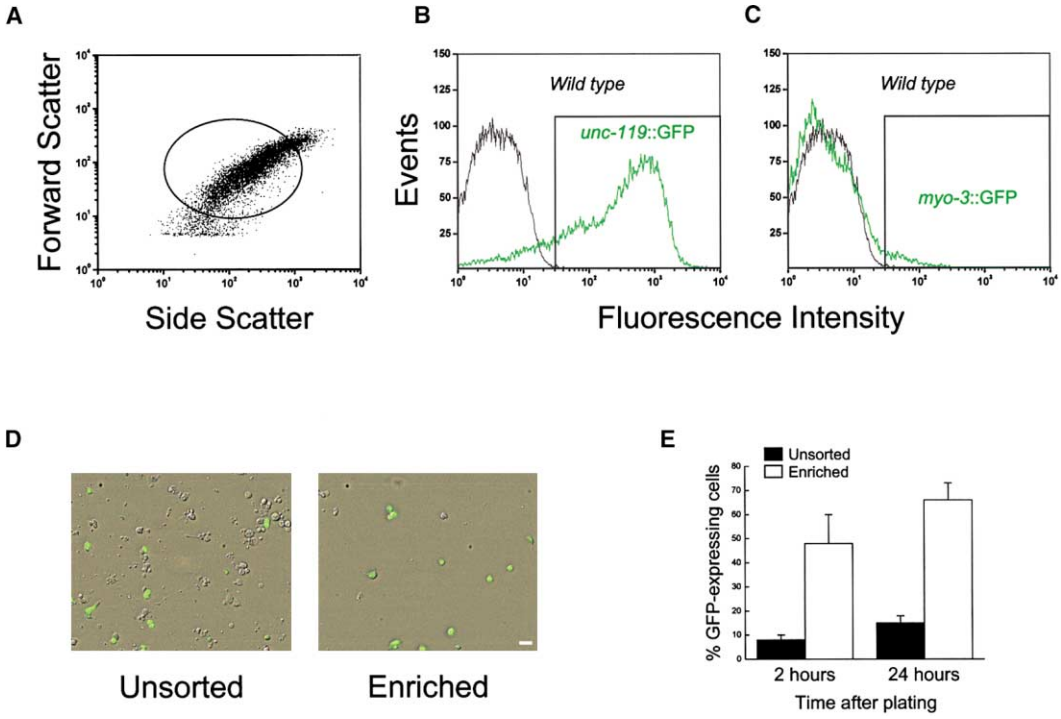

Figure 4. Flow Cytometry and Culture of Embryonic Cells

(A) Forward (size) versus side (granularity) scatter plot of wild-type embryonic cells. The outlined area indicates cells that were analyzed for GFP fluorescence.

(B and C) Comparison of fluorescence intensity of embryonic cells isolated from the wild-type and from *unc-119*::GFP and *myo-3*::GFP transgenic lines. Cells within the boxed region showed fluorescence intensities greater than 99.9% of the wild-type cells and were therefore deemed GFP-positive. GFP-expressing cells represented 87% and 4% of the total embryonic cells analyzed from *unc-119*::GFP and *myo-3*::GFP strains, respectively.

(D) Combined DIC and fluorescence micrographs of representative fields of unsorted cultures and cultures enriched for *myo-3*::GFP. Micrographs were obtained 24 hr after plating. Scale bar is 10 μm.

(E) Percentage of total number of cells expressing GFP in unsorted and *myo-3*::GFP-enriched cultures. Cells in random fields were counted at 2 and 24 hr following sorting and plating. Values are means ± SD (n = 3). Total number of cells counted was >13,000 for unsorted cultures and >1,600 for FACS-enriched cultures.

mimic those observed postembryonically. Additional studies are needed to address this possibility.

Isolation of GFP-Expressing Cells by Fluorescence-Activated Cell Sorting

Our finding that embryonic cells express specific GFP markers in vitro offers the possibility of using FACS to generate enriched populations of differentiated *C. elegans* cells for biochemical and molecular analyses. To test this idea, embryonic cells were isolated from wild-type and GFP transgenic lines. Figure 4A shows a forward versus side scatter plot of freshly isolated wild-type embryonic cells. Similar profiles were obtained for GFP transgenic lines (data not shown). As expected, wild-type embryonic cells vary in size (i.e., forward scatter). GFP fluorescence intensity was analyzed in a population of cells with a median size range (outlined area in Figure 4A). Of these cells, 0.1% exhibited significant fluorescence levels, which we define as background autofluorescence.

Approximately 87% of the embryonic cells obtained from the *unc-119*::GFP line exhibit bright fluorescence (enclosed with black line; Figure 4B). The FACS profile of the *unc-119*::GFP culture is indicative of a range of cell sizes and GFP fluorescent intensities. This heterogeneity was confirmed by fluorescence microscopy (data not shown) and is expected from the in vivo expression of *unc-119*::GFP in all neurons and their embryonic precursors (Maduro and Pilgrim, 1995). The *myo-3*::GFP transgenic line yields fewer GFP-expressing cells (~4%), as predicted from the selective expression of *myo-3* in body muscle cells in vivo (Ardizzi and Epstein, 1987; Miller et al., 1983).

Sorted *myo-3*::GFP-expressing cells were collected and placed in culture. Trypan blue exclusion demonstrated that >95% of the sorted cells were viable (data not shown). Cultures produced from the sorted and unsorted cells were analyzed by light microscopy. Within 2 hr of plating, 8% ± 2% of cells in the unsorted cultures expressed GFP, whereas 48% ± 12% of the FACS-enriched cultures showed GFP fluorescence (Figures 4D and 4E).

The fraction of *myo-3*::GFP cells in unsorted cultures increased significantly ($p < 0.03$; unpaired Student's t test) within 24 hr of plating (Figure 4E). Similarly, *unc-4*::GFP-expressing cells were rare 1 hr after isolation (~0.2%), but increased to ~3% of all cells observed in 24 hr cultures (data not shown). The increase in the

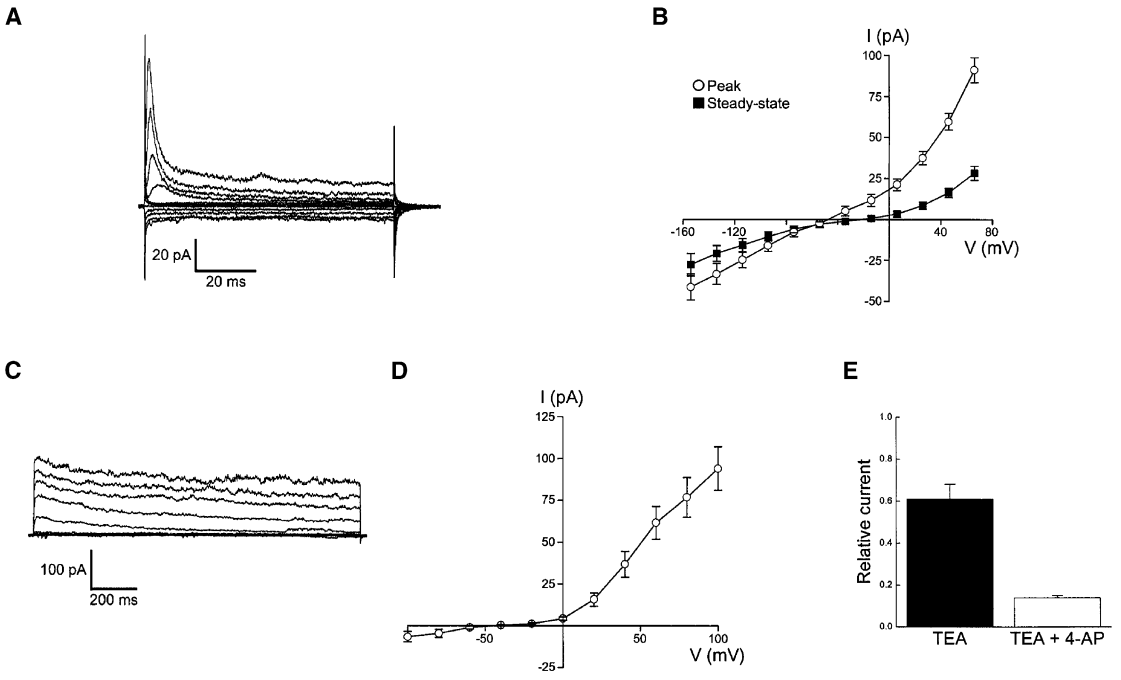

Figure 5. Electrophysiological Properties of Cultured ASER Chemosensory Neurons and Body Wall Muscle Cells

(A) Whole-cell currents in a cultured *gcy-5*::GFP-expressing ASER neuron elicited by stepping membrane potential from a holding potential of −74 mV to values of −154 to +66 mV in 20 mV steps.

(B) Current versus voltage relationship of peak and steady-state ASER neuron whole-cell currents. Mean steady-state current was measured over the last 10 ms of the voltage step. Values are means ± SE (n = 14).

(C) Whole-cell currents in a cultured *myo-3*::GFP-expressing body wall muscle cell. Currents were elicited by stepping membrane potential from a holding potential of −80 mV to values of −100 to +100 mV in 20 mV steps.

(D) Current versus voltage relationship of steady-state muscle cell currents measured as the mean current observed during the last 250 ms of the voltage step. Values are means ± SE (n = 8).

(E) Inhibitory effects of bath addition of 20 mM TEA or 20 mM TEA and 3 mM 4-aminopyridine (4-AP). Steady-state currents in the presence and absence of the drugs were measured at +80 mV. Drug effects were reversible (data not shown). Values are means ± SE (n = 3–5).

frequency of *unc-4*::GFP cells with time in culture was also observed by time lapse imaging (see Supplemental data at http://www.neuron.org/cgi/content/full/33/4/503/DC1). These additional muscle cells and motor neurons are likely to arise from mitotic precursors in culture. Flow cytometry of propidium iodide-treated cells demonstrated that ~30% of freshly prepared embryonic cells are either in S phase or actively dividing (G_2/M). The proportion of cells undergoing mitosis drops to 4% ± 2% (n = 2) after 24 hr in culture (see Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/33/4/503/DC1). BrdU labeling also demonstrated that at least some precursor cells give rise to neuronal and muscle-like cells (see Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/33/4/503/DC1). Taken together, our findings indicate that neurons and muscle cells proliferate as well as differentiate in culture

Functional Characterization of Cultured Neurons and Muscle Cells

We undertook a series of patch clamp studies to assess the feasibility of in vitro electrophysiological analysis of defined cell types and to determine whether the functional properties of cultured cells resemble those observed in vivo. ASER neurons express the guanylyl cyclase gene *gcy-5* (Yu et al., 1997). We cultured cells from the *gcy-5*::GFP transgenic line. GFP-positive neurons were patch clamped in the conventional whole-cell mode. As shown in Figure 5, ASER neurons express rapidly activating and inactivating, outward whole-cell currents and time-independent inward currents. Outward current inactivated with a mean ± SE time constant of 11.3 ± 1.4 ms (n = 15). The inactivation rate constant and other biophysical characteristics of the currents in cultured ASER neurons are similar to those described by Lockery and coworkers (Goodman et al., 1998; Pierce-Shimomura et al., 2001). Ion substitution and pharmacological experiments performed in vivo (Goodman et al., 1998) demonstrated that the outward current is carried by K^+ channels.

The recent development of a so-called "filleted worm" preparation allows physiological access to body wall muscles. Experiments with this preparation defined the roles of GABA and acetylcholine receptors at the neuromuscular junction (Richmond and Jorgensen, 1999) and the involvement of *unc-13* in synaptic vesicle exocytosis (Richmond et al., 1999). As shown in Figure 5, cultured muscle cells express slowly inactivating, outwardly rectifying currents. These currents were inhibited ~40% by 20 mM TEA and ~85% by 20 mM TEA plus 3 mM 4-aminopyridine (4-AP). The characteristics of the outward currents observed in vitro are similar to those de-

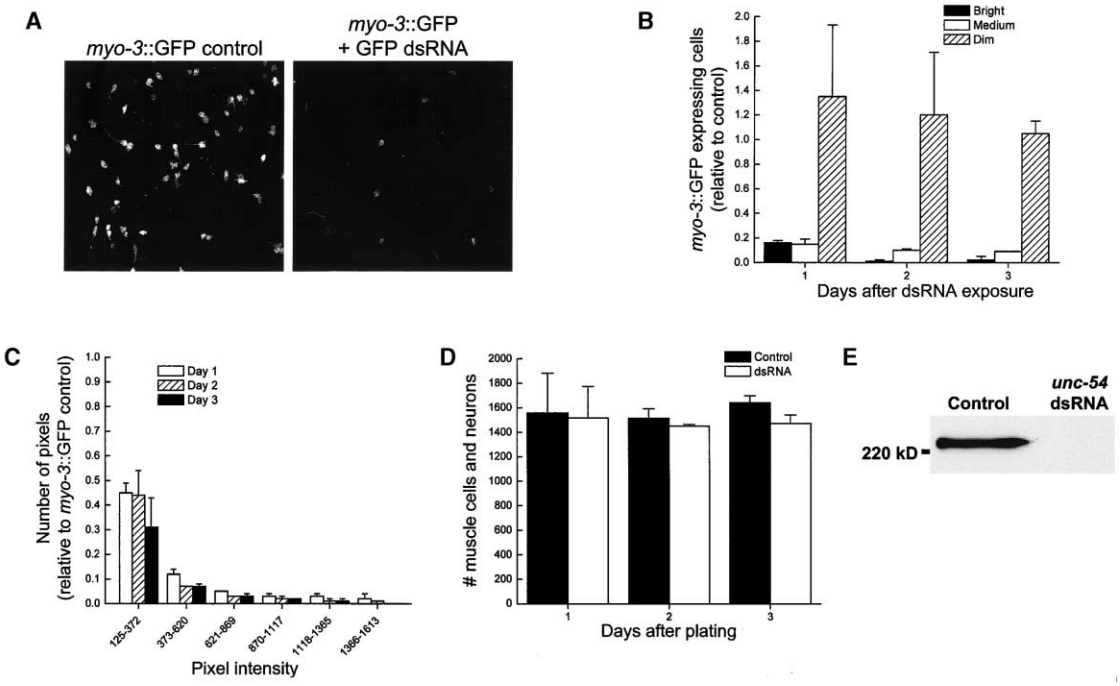

Figure 6. Effect of GFP dsRNA on GFP Levels in *myo-3*::GFP-Expressing Muscle Cells

(A) Fluorescence micrographs of control *myo-3*::GFP cells and cells treated with GFP dsRNA for 3 days. Analysis of paired brightfield images demonstrated that 123 and 129 muscle cells and neurons were present in the fields from the control and dsRNA-treated cultures, respectively.

(B) Relative number of *myo-3*::GFP-expressing cells in cultures treated with GFP dsRNA. GFP fluorescence in single cells was scored as bright, medium, or dim. Imaging protocol was the same as described in (A). Values are means ± SD of two independent experiments.

(C) Pixel intensities (relative to control) of images of *myo-3*::GFP-expressing cells treated with dsRNA for 1–3 days. Images were obtained daily for three successive days from 16 random fields visualized in paired control and dsRNA-treated cell cultures. Values are means ± SD of two independent experiments.

(D) Total number of muscle cells and neurons present in micrographs obtained from control and dsRNA-treated cell cultures. Cells were exposed to dsRNA immediately after plating. Imaging protocol was the same as described in (A). Values are means ± SD of two independent experiments.

(E) Western blot illustrating the effect of *unc-54* dsRNA on UNC-54 expression in cultured embryonic cells. Cells were treated with dsRNA for 4 days before protein extraction and Western blotting.

scribed for body wall muscles patch clamped in vivo (Richmond and Jorgensen, 1999).

Although inward, voltage-dependent Ca^{2+} currents have been observed in body wall muscle in vivo (Richmond and Jorgensen, 1999), we were unable to detect whole-cell Ca^{2+} currents in cultured muscle cells. It is possible that expression of Ca^{2+} channels requires functional interactions with other cell types. Alternatively, Ca^{2+} channel expression in single muscle cells may be too low to detect Ca^{2+} currents at the whole-cell level. Muscle cells are coupled by gap junctions in vivo (White et al., 1986), and whole-cell measurements on filleted worm preparations record currents from a syncitium comprised of multiple cells.

Double-Stranded RNA Disrupts Targeted Gene Expression in Cultured Embryonic Cells

Double-stranded RNA (dsRNA)-mediated gene interference (RNA_i) is a potent and highly selective tool for disrupting gene expression in invertebrate animals, plants, and protozoa (Bosher and Labouesse, 2000; Zamore, 2001). RNA_i has also been demonstrated to work effectively in *Drosophila* S2 cell lines (Caplen et al., 2000; Clemens et al., 2000; Ui-Tei et al., 2000). We therefore undertook a series of studies to assess the ability of dsRNA to disrupt gene expression in cultured *C. elegans* cells.

Figure 6A shows a typical fluorescence micrograph of control *myo-3*::GFP-expressing cells and cells treated with GFP dsRNA for 3 days. GFP levels were quantified by scoring cells as expressing bright, medium, or dim GFP fluorescence. As shown in Figure 6B, the number of cells exhibiting bright and medium GFP fluorescence was reduced by 85%–90% (Figures 6B and 6C) within 24 hr of treatment with dsRNA.

GFP levels were also quantified by measuring the intensity of all pixels in each fluorescence image. As shown in Figure 6C, there was a 50% to >90% reduction in the number of pixels within the measured intensity ranges in images of cells treated with dsRNA. This effect was maximal within 24 hr after dsRNA exposure.

To ensure that the reduction in GFP fluorescence was not due to a loss of cells in the dsRNA-treated cultures, we also counted the total number of cells in each field that had muscle and neuronal morphology. As shown in Figure 6D, the number of muscle cells and neurons in both control and dsRNA-treated cultures was similar.

We also examined the effect of dsRNA on the expres-

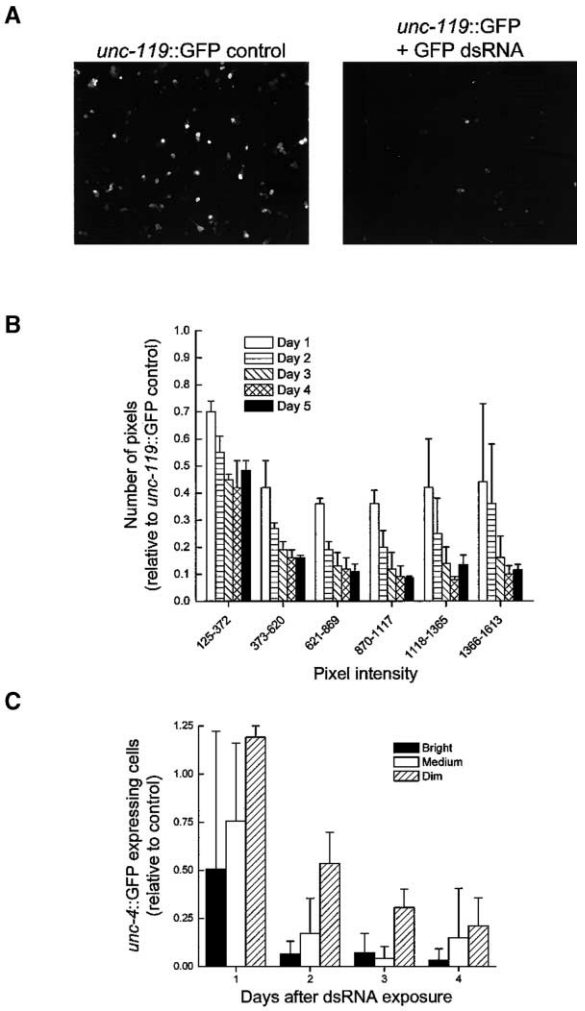

Figure 7. Effect of dsRNA on Gene Expression in Cultured Neurons

(A) Fluorescence micrographs of control *unc-119*::GFP neurons and neurons treated with GFP dsRNA for 4 days.

(B) Pixel intensities (relative to control) of images of *unc-119*::GFP-expressing cells treated with dsRNA for 1–5 days. Images were obtained from 16 random fields visualized in paired control and dsRNA-treated cell cultures.

(C) Relative number of *unc-4*::GFP-expressing neurons in cultures treated with GFP dsRNA. GFP fluorescence in single cells was scored as bright, medium, or dim. Imaging protocol was similar to that described in (B). Values are means ± SD of 2–3 experiments.

sion of the native, myosin-encoding gene *unc-54* (Epstein et al., 1974; Miller et al., 1983). Figure 6E shows an UNC-54 Western blot of proteins obtained from control cells and cells exposed to *unc-54* dsRNA for 4 days. UNC-54 is virtually undetectable in the dsRNA-treated cells. The mean dsRNA-induced reduction of UNC-54 expression was 94 ± 3% (mean ± SE; n = 3).

To assess the effectiveness of RNA_i on neurons in vitro, we monitored GFP expression in cultures derived from the *unc-119*::GFP worms. As shown in Figures 7A and 7B, GFP dsRNA dramatically reduced GFP expression in cultured neurons. However, unlike *myo-3*::GFP, downregulation of *unc-119*::GFP was considerably slower. Knockdown of GFP expression in muscle cells was largely complete one day after exposure to dsRNA (Figure 6A). In contrast, reduction of *unc-119*::GFP appeared to be maximal 4 days after dsRNA treatment (Figure 7B). The slower reduction of GFP expression in dsRNA-treated cultured neurons is consistent with in vivo observations (Timmons et al., 2001).

The effectiveness of dsRNA on neuronal gene expression was examined further by monitoring GFP fluorescence in cells cultured from *unc-4*::GFP transgenic worms. *unc-4*-expressing cells were scored manually as expressing bright, medium or dim GFP fluorescence. As shown in Figure 7C, GFP fluorescence levels were only modestly affected 1 day after dsRNA exposure. However, 3 days after treatment with dsRNA, there was a >90% reduction in the number of cells expressing bright and medium GFP fluorescence levels.

While our results demonstrate clearly the effectiveness of RNA_i in disrupting the expression of GFP and UNC-54 in cultured *C. elegans* muscle cells and neurons, it is conceivable that other genes and/or cell types may be less sensitive to dsRNA. Clearly, it will be important for investigators using this approach to assess both the time course and extent of gene expression knockdown by dsRNA in their specific experimental setting.

Discussion

Although cell culture systems have been widely utilized for other organisms, robust methods for mass culture of *C. elegans* cells have not been previously available. We have now developed culture conditions in which *C. elegans* embryonic cells undergo extensive differentiation within 24 hr of isolation. The availability of cell-specific GFP reporter genes allows the ready identification and isolation of cell types from this preparation (Figure 2). Cultured cells survive well in vitro for many days to weeks (Figure 1).

An important concern for primary cell culture experiments is that in vitro conditions may alter cell fates and thus limit the utility of this approach for understanding cell differentiation and function in the intact animal. Disruption of the *C. elegans* embryo, for example, effectively displaces the isolated blastomeres from adjacent cells that might provide cues essential to normal differentiation. Indeed, key cell fate decisions in the early *C. elegans* embryo are crucially dependent on intercellular signaling pathways (Schnabel and Priess, 1997). As embryogenesis proceeds, however, many *C. elegans* embryonic cells assume a cell-autonomous developmental potential (Mello et al., 1992; Priess and Thomson, 1987). For example, an isolated E blastomere, the embryonic precursor to all intestinal cells, is capable of generating a polarized epithelial structure in vitro comprised exclusively of gut-like cells (Leung et al., 1999)

We have now shown that embryonic cells giving rise to muscles and specific neurons also appear to differentiate normally in vitro. Several lines of evidence support this conclusion. First, cultures derived from nematode strains expressing specific GFP reporters produce GFP-labeled cells that are morphologically similar to these cells in the intact animal (Figure 2). Second, the proportions of GFP-expressing cells that appear in vitro are predicted by the abundance of these cells in the mature embryo (Table 1). Third, specific markers of differentia-

tion are coexpressed in identified neurons and muscle cells in culture (Figure 2). Fourth, electrophysiological properties of at least two classes of excitable cells, ASER sensory neurons and body muscles, resemble measurements obtained in vivo (Figure 5).

It seems likely that a wide variety of cell types may retain the capacity to differentiate in culture. In each case, however, it will be necessary to assess the extent to which these cells adopt the expected array of molecular and functional traits. The existing detailed knowledge of *C. elegans* embryonic development (Sulston et al., 1983) coupled with the availability of a variety of cell-specific GFP markers should lead to the rapid evaluation of the reliability of the culture method for studies of these additional classes of cells.

Primary nematode cell culture provides a new foundation for a broad array of experimental opportunities heretofore unavailable in the field. For example, detailed electrophysiological characterization of somatic cells in *C. elegans* has been difficult due the presence of a tough exoskeleton. However, cultured cells are readily accessible for patch clamp studies (Figure 5). The only technical hindrance for patch clamp investigations is the relatively small size of the cells. Despite this, we have found that gigaohm seals and whole-cell access are straightforward to obtain on muscle cells and ASER neurons as well as several other cell types such as body "touch" neurons (Christensen and Strange, 2002) and intestinal cells (A.E. and K.S., unpublished data). Cultured cells can also be readily loaded with fluorescent probes that measure the levels of intracellular ions such as Ca^{2+} and H^{+} (A.E. and K.S., unpublished data). The combination of direct physiological measurements on cells from wild-type and mutant animals with reverse genetic strategies such as RNA_i (Figures 6 and 7) should provide unique molecular insights into cell excitability, intracellular signaling, and ion channel function and regulation, as well as other important cellular processes.

Identified cell types can be isolated from cultured embryonic cells using FACS methods (Figure 4). These enriched cell populations will be valuable for cell-specific molecular studies and for production of cell-specific cDNA (R.F., M.Mc., D.M.M., unpublished data). Cell culture used in combination with sorting methods and microarray and proteomic analyses provides powerful strategies to define cell-specific gene expression patterns under control conditions, after experimental perturbations, and in mutant animals. For example, Chalfie and coworkers have identified touch-cell specific genes by hybridizing a *C. elegans* microarray with amplified mRNA from FACS-enriched GFP-expressing touch neurons. (Y. Zhang, C. Ma, T. Delohery, B. Nasipak, S.K. Kim, and M. Chalfie, personal communication).

C. elegans is a powerful model system for identifying genes involved in synaptogenesis, growth cone motility, and axon guidance (Merz and Culotti, 2000). We have observed contacts between neurons and between neurons and muscle cells that could be indicative of synapse formation in culture (Figure 2F). Although we have not conducted experiments to confirm this possibility directly, neurons and muscle cells isolated from numerous various vertebrate and invertebrate species are capable of establishing functional synapses in vitro (Bi and Poo, 1998; Schmidt et al., 2000). Moreover, in many instances, these cells retain synaptic specificity for their in vivo targets (Camardo et al., 1983; Yamamoto et al., 1992). Although ultrastructural analysis has cataloged the synapses between all 302 neurons in the *C. elegans* nervous system (White et al., 1986), the electrophysiologic properties of most of these connections have not been evaluated. In principle, many of these synapses should now be accessible for the first time for functional analysis in vitro. The availability of multicolor GFP markers for specific cell types (Miller et al., 1999) and cell sorting methods will facilitate coculture experiments.

Signaling mechanisms that control axon motility and guidance in vitro could be characterized by manipulating the concentrations of extracellular signaling molecules such as UNC-6/netrin (Merz and Culotti, 2000) and by disrupting gene expression with RNA_i in cultures prepared from control and mutant animals. The role of intracellular signals such as Ca^{2+} (Gomez et al., 2001) and cAMP (Song et al., 1997) in regulating guidance events can be investigated using fluorescent probes sensitive to these molecules and by altering their cytoplasmic concentrations using conventional experimental approaches.

The exploitation of powerful genetic, cell biological, and functional genomics approaches has led to a detailed description of *C. elegans* biology and development. However, the relative inaccessibility of *C. elegans* somatic cells has largely prevented in depth biochemical and physiological studies of specific cell types. The primary cell culture methods we describe here largely circumvent this barrier and, in combination with existing *C. elegans* tools, provide a new opportunity for developing an integrated systems level understanding of eukaryotic biology.

Experimental Procedures

C. elegans Strains

All strains were derived from the wild-type N2 line and maintained at 20°C–25°C utilizing standard methods (Brenner, 1974). The strains used in these studies were: *unc-119*::GFP DP132 (*edIs6 IV*) (Maduro and Pilgrim, 1995); *unc-4*::GFP NC197 (*wdIs4 II*) (Pflugrad et al., 1997); *opt-3*::GFP KM173 (*opt-3*::GFP pRF4) (Fei et al., 2000); *myo-3*::GFP PD4251(*ccIs4251 I*) (Fire et al., 1998); *gcy-5*::GFP DA1262 (*adEx1262*) (Yu et al., 1997); *acr-5*::YFP *unc-4*::CFP NC582 (*wdIs19; wdIs23*) this work; *mec-4*::GFP (*zdIs4*); and *mec-7*::GFP CF702. All GFP strains contain integrated transgenes with the exception of the *gcy-5*::GFP line, which carries an extrachromosomal array.

Embryonic Cell Isolation

Cultures of synchronized adults were prepared as described previously (Lewis and Fleming, 1995). Adults were lysed by exposing them to 0.5 M NaOH and 1% NaOCl. The lysis reaction was stopped after 5 min by diluting the lysis solution two times with sterile egg buffer containing 118 mM NaCl, 48 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 25 mM HEPES (pH 7.3, 340 mOsm) (Edgar, 1995). Eggs released by this treatment were pelleted by centrifugation and then washed three times with sterile egg buffer. Adult carcasses were separated from washed eggs by centrifugation in sterile 30% sucrose. The egg layer was removed by pipette and washed one time with sterile water and then one time with sterile egg buffer.

Embryo isolation, cell dissociation, and cell plating were carried out in a laminar flow hood. Eggshells were removed by resuspending ~25–100 μl of pelleted eggs in a sterile 1.7 ml microfuge tube with 0.5 ml egg buffer containing 1 U/ml of chitinase (Sigma Chemical, St. Louis, MO). Egg suspensions were rocked gently at room temperature for 20–40 min. Because the activity of different lots of chitinase varies substantially, eggshell digestion was monitored periodically

by examining aliquots of the suspension on a dissecting microscope.

When approximately 80% of the eggshells were digested, embryos were pelleted by brief centrifugation, and the egg buffer was removed and replaced with 800 μl of L-15 cell culture medium (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (HYCLONE, Logan, UT), 50 U/ml penicillin, and 50 μg/ml streptomycin. The osmolality of the culture medium was adjusted to 340 mOsm with sucrose and filter sterilized.

Embryos were dissociated by gentle pipetting. The extent of dissociation was monitored periodically by examining aliquots of the embryo suspension on a dissecting microscope. After dissociation was complete, cells were pelleted and resuspended in 500–600 μl of cell culture medium. Intact embryos, clumps of cells, and larvae were removed from the cell suspension by filtration. Briefly, 1 ml of cell culture medium was drawn up into a 3 ml sterile syringe through an 18G needle. The cell suspension was then drawn gently up into the syringe, and this mixture was filtered through a 5 μm Durapore syringe filter (Millipore, Bedford MA). An additional 1 ml of culture medium was flushed through the filter to remove adherent cells. Filtered cells were pelleted and resuspended in cell culture medium to a final stock concentration of approximately 10–20 $\times$ 10^6 cells/ml.

Visual inspection of dissociated embryo preparations prior to filtration indicated that cells were isolated predominately from pre-"comma" stage embryos. Propidium iodide and GFP reporter expression studies support this conclusion. Approximately 30% of freshly isolated cells are either mitotic or in S phase (see Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/33/4/503/DC1). These cells likely arise from early embryos, as cell division stops in vivo after the comma stage (Sulston et al., 1983). The frequency of *unc-119*::GFP and *myo-3*::GFP expression in freshly isolated cells (Table 1 and Figure 4) is similar to that observed in precomma stage embryos (Gossett et al., 1982; Maduro and Pilgrim, 1995). In addition, *unc-4*::GFP, which is not expressed until after the comma stage (Miller and Niemeyer, 1995), is rare in freshly isolated cells, but increases significantly within the first 24 hr in culture (Table 1 and see Supplemental data at http://www.neuron.org/cgi/content/full/33/4/503/DC1).

Cell Culture

Cells were cultured on 1-well chamber slides (Nalge Nunc International, Naperville, IL), 4- or 8-well chambered coverglasses (Nalge Nunc International), or acid-washed 12 mm diameter glass cover slips. In order for cell morphological differentiation to take place, cells must adhere to the growth substrate. Most of the experiments described in this paper were carried out using peanut lectin (Sigma) to promote cell adhesion (Buechner et al., 1999). Peanut lectin was dissolved in sterile water at a concentration of 0.5 mg/ml. Glass growth surfaces were coated with the lectin solution for 10–20 min. The solution was then removed and the growth surfaces allowed to air dry in a laminar flow hood under UV light. Lectin-coated growth surfaces were stored for weeks under sterile conditions without apparent degradation of the lectin coating.

Cells were typically seeded at densities of 120,000–375,000 cells/cm^2 in L-15 cell culture medium. Cultures were maintained at 20°C–25°C in a humidified incubator. To minimize evaporation, culture vessels were placed in plastic boxes lined with wet paper towels.

RNA Interference

Double-stranded RNA (dsRNA) was synthesized using established methods (Fire et al., 1998). Briefly, a DNA template encoding nucleotides 5236–5851 of *unc-54* mRNA was obtained by RT-PCR (Miller and Niemeyer, 1995). The vector pPD79.44 was used for GFP dsRNA synthesis. Sense and antisense RNA were synthesized by T3 and T7 polymerase reactions (MEGAscript kit, Ambion, Austin, TX). Template DNA was digested with DNaseI and RNA purified by ethanol precipitation. dsRNA was formed by dissolving purified RNA in RNase-free water and then heating to 65°C for 30 min followed by cooling to room temperature. The size, purity, and integrity of dsRNA were assayed on TAE agarose gels.

Equal numbers of cells from a cell isolate were plated in either control L-15 cell culture medium or L-15 medium containing 15 μg/ml dsRNA. Two to three hours after plating, the dsRNA was diluted to a final concentration of 5 μg/ml. Gene expression was quantified in parallel in both control and dsRNA-treated cells and is expressed relative to that observed in the control cultures.

Microscopy

Cells were observed by brightfield, differential interference contrast (DIC), or epifluorescence microscopy. Images were recorded using high resolution, cooled CCD cameras (MicroMax, Princeton Instruments, Trenton, NJ; ORCA I, ORCA ER, Hamamatsu Corporation, Bridgewater NJ). Quantification of pixel intensity was performed with MetaMorph software (Universal Imaging, West Chester, PA).

Western Analysis

Cells were cultured in 1-well chamber slides at a density of approximately 850,000 cells/cm^2. Four days after plating, cells were lysed in PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Molecular Biochemicals, Indianapolis, IN). The protein content of cell lysates was determined by BCA-200 protein assay (Pierce, Rockford, IL). Twenty-five micrograms of total protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. UNC-54 was visualized by Western analysis using a monoclonal anti-UNC-54 antibody mAb 5-8 (Miller et al., 1986) and SuperSignal Substrate reagents (Pierce). UNC-54 expression was quantified by digitizing blots and measuring optical density using NIH Image software.

Immunofluorescence

Cells were fixed with 1% paraformaldehyde for 30 min, rinsed, and then permeabilized for 2 min with ice-cold methanol. Permeabilized cells were incubated for 60 min with UNC-54 mAb 5-8 (Miller et al., 1983) or with synaptotagmin (SNT-1) (Lickteig et al., 2001) antibody followed by incubation for 30 min with Cy3-labeled secondary antibodies.

Creation of Transgenic Animals

GFP coding sequence was removed from the *acr-5*::GFP plasmid, pJR7, by cutting with AgeI and ApaI and replaced with the corresponding YFP region from pPD132.112. An *acr-5*::YFP transgenic line was generated by coinjection with the *rol-6* dominant marker, pRF4 (Mello and Fire, 1995). *acr-5*::YFP and *unc-4*::CFP transgenic arrays (Miller et al., 1999) were chromosomally integrated by X-ray treatment and intercrossed to produce a strain expressing both transgenes.

FACS Analysis

Embryonic cells from wild-type or GFP-expressing lines were dispersed in egg buffer. FACS analysis and sorting were performed using a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon laser and FITC filter set. *myo-3*::*GFP* cells were collected by low speed centrifugation following sorting, resuspended in L-15 medium with 10% FBS, and seeded onto peanut lectin-coated chambered coverglasses.

Analysis of Cell Cycle and Cell Division

(See supplemental data at http://www.neuron.org/cgi/content/full/33/4/503/DC1)

Patch Clamp Electrophysiology

Cells were cultured on 12 mm diameter glass coverslips. The coverslips were attached to the bottom of a bath chamber (model R-26G; Warner Instrument, Hamden, CT) mounted onto the stage of a Nikon inverted microscope, and cells were visualized by video-enhanced DIC and fluorescence microscopy. Patch electrodes were pulled from 1.5 mm outer diameter silanized borosilicate microhematocrit tubes. Currents were measured with Axopatch 200 (Axon Instruments, Foster City, CA) patch clamp amplifiers. Electrical connections to the amplifier were made using Ag/AgCl wires and 3 M KCl/agar bridges. Data acquisition was performed using pClamp 6 software (Axon Instruments). Bath and pipette solutions used for patch clamp measurements on cultured *myo-3*::GFP-expressing muscle cells and *gcy-5*::GFP-expressing ASER chemosensory neurons were identical to those described by Richmond and Jorgensen

(Richmond and Jorgensen, 1999) and Lockery and coworkers (Lockery and Goodman, 1998; Goodman et al., 1998; Pierce-Shimomura et al., 2001), respectively.

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