Boundary Formation by Notch Signaling in Drosophila Multicellular Systems: Experimental Observations and Gene Network Modeling by Genomic Object Net

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BOUNDARY FORMATION BY NOTCH SIGNALING IN DROSOPHILA MULTICELLULAR SYSTEMS: EXPERIMENTAL OBSERVATIONS AND GENE NETWORK MODELING BY GENOMIC OBJECT NET

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The Delta-Notch signaling system plays an essential role in various morphogenetic systems of multicellular animal development. Here we analyzed the mechanism of Notch-dependent boundary formation in the *Drosophila* large intestine, by experimental manipulation of Delta expression and computational modeling and simulation by Genomic Object Net. Boundary formation representing the situation in normal large intestine was shown by the simulation. By manipulating Delta expression in the large intestine, a few types of disorder in boundary cell differentiation were observed, and similar abnormal patterns were generated by the simulation. Simulation results suggest that parameter values representing the strength of cell-autonomous suppression of Notch signaling by Delta are essential for generating two different modes of patterning: lateral inhibition and boundary formation, which could explain how a common gene regulatory network results in two different patterning modes in vivo. Genomic Object Net proved to be a useful and flexible biosimulation system that is suitable for analyzing complex biological phenomena such as patternings of multicellular systems as well as intracellular changes in cell states including metabolic activities, gene regulation, and enzyme reactions.

1 Introduction

Pattern formation of multicellular organisms includes intracellular regulatory events such as gene activation/repression, enzymatic reactions generating/degrading various kinds of biomolecules, as well as cell-to-cell interactions that coordinate intracellular events of individual cells.

The Delta-Notch signaling pathway plays an essential role in various morphogenetic systems of multicellular animal development. Lateral inhibition through Delta-Notch signaling pathway was examined during the emergence of ciliated cells in *Xenopus* embryonic skin [1]. Ghosh and Tomlin [2] modeled

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the Delta-Notch signaling pathway as a hybrid system and presented results in both simulation and reachability analysis of this hybrid system. Bockmayr and Courtois [3] gave an another hybrid system approach, hybrid concurrent constraint programming, for modeling dynamic biological systems including the Delta-Notch signaling pathway. In their methods, to analyze a biological system, it has to be translated into a complete set of mathematical formulas for simulation. However, this task is in practice difficult for most biologists. In contrast, we have proposed a new method for modeling biological phenomena, which is based on the hybrid Petri net (HPN) [4]. In this approach, we only need to diagrammatize known or hypothetical biological pathways, without writing mathematical formulas, and define simple rules for the kinetics of each biomolecular component.

We have been developing a biosimulation system, Genomic Object Net (GON), whose architecture is essentially based on the hybrid functional Petri net (HFPN) and XML technology. The HFPN [5] was introduced by extending the notion of HPN [6] so that various aspects in biopathways can be modeled smoothly while inheriting good traditions from the the research on Petri net [7]. With GON, we have modeled and simulated many biopathways including the gene switch mechanisms of λ phage [4,8,18], the gene regulation for circadian rhythm in *Drosophila* [9,18], the signal transduction pathway for apoptosis induced by the protein Fas [9,18], and the glycolytic pathway in *E.coli* with the *lac* operon gene regulatory mechanism [5,18].

As a next step for exploiting GON, we here present a method to model a multicellular patterning system by HFPN with a novel visualizing function suitable for monitoring the simulation process of multicellular systems. In this paper, we analyzed the mechanism of Notch-dependent patterning events in the *Drosophila* large intestine, by combining experiments on live materials and computational modeling by GON.

2 Outline of the Delta-Notch signaling pathway

Cell-to-cell interactions mediated by Notch signal transduction pathway play essential roles in development of a multicellular organism [10]. Both of Delta and Notch proteins are initially expressed as membrane proteins. In canonical Notch signaling pathway, Delta binds to inactive Notch protein of adjacent cells, and triggers activation of Notch. After a few steps of activation, the intracellular domain of Notch is released by proteolytic cleavage and becomes active. The active form of Notch causes a change in gene expression pattern, including a down-regulation of *Delta* gene expression [10]. In addittion to the activation of Notch protein of adjacent cells, Delta has a suppressive effect

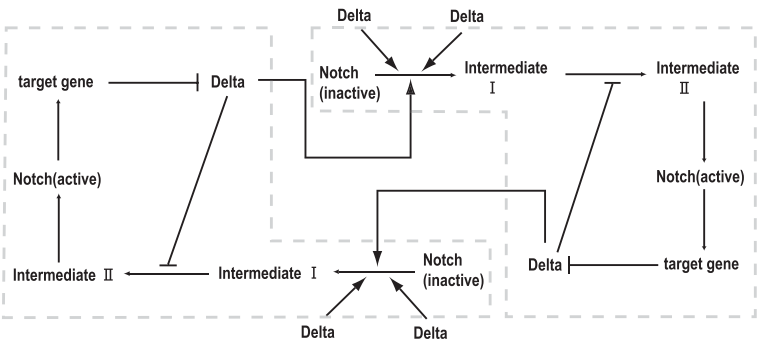


Figure 1. Two cells model of Delta-Notch Signaling pathway. Notch activation process by Delta of adjacent cells as well as the cell-autonomous suppression of Notch signaling by Delta in Delta-positive cells is included. Arrows and bars in the pathway represent activation and suppression, respectively.

on Notch signaling within Delta-positive cells [11,12]. This is a core feature of the Delta-Notch pathway common to various patterning systems of the multicellular animal development (Figure 1). These feedback loops, an essential feature of the Delta-Notch system, often make it difficult to predict how the system works in vivo. Furthermore, in spite of a seemingly simple core regulatory pathway, Notch signaling causes various types of pattern formation, depending on the developmental system in which the Notch pathway is working. One of the most well-known example of Notch-dependent patterning is the lateral inhibition [10], in which one cell is singled out from a group of equivalent precursor cells. The other example is the boundary formation between two different fields of cells [10]. In the present study, we analyzed the mechanism of the boundary cell formation in the *Drosophila* large intestine, in which a single row of boundary cells is induced between dorsal and ventral domains [13,14,15,16].

3 Hybrid Functional Petri Net Modeling of the Delta-Notch Pathway

3.1 Basic Elements

In general, a *Petri net* [7] is a network consisting of the following four kinds of elements: place, transition, arc, and token. A *place* holds *tokens* as its content. A *transition* has arcs coming from places and arcs going out from

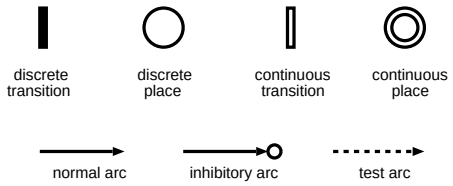


Figure 2. Basic elements of hybrid (functional) Petri net.

the transition to some places. A transition with these arcs defines a *firing rule* in terms of the contents of the places where the arcs are attached.

In *hybrid Petri net* (HPN) model [6], two kinds of places and transitions are used, *discrete/continuous places* and *discrete/continuous transitions*. A discrete place and a discrete transition are the same notions as used in the traditional *discrete Petri net* model [7]. A continuous place holds a nonnegative real number as its content. A continuous transition fires continuously in the HPN model and its firing speed is given as a function of values in the places in the model. The graphical notations of a discrete transition, a discrete place, a continuous transition, and a continuous place are drawn in Figure 2, together with three types of arcs. The same basic elements in Figure 2 are used in HFPN. Refer to reference [5] for the details of HFPN.

Specific values are assigned to each arc as a weight. When a *normal arc* with weight w is attached to a discrete transition, w tokens are transferred through the normal arc. On the other hand, when a normal arc is attached to a continuous transition, the amount of token that flows is determined by the firing speed of the continuous transition. An *inhibitory arc* with weight w enables the transition to fire only if the content of the place at the source of the arc is less than or equal to w. For example, an inhibitory arc can be used to represent repressive activity in gene regulation. A *test arc* does not consume any content of the place at the source of the arc by firing. For example, test arcs can be used to represent enzyme activity, since the enzyme itself is not consumed.

3.2 Modeling the Delta-Notch Signaling Pathway

The Delta-Notch pathway depicted in Figure 1 is modeled by an HFPN, which includes the intracellular regulatory circuit as well as cell-to-cell interactions (Figure 3). In Figure 3, an HFPN model of the complete intracellular circuit of a single Cell A, with interactions with adjacent Cells B and C, is illustrated.

When the amount of Delta in Cell B (Cell C) exceeds level 1, token value is transferred from the place Notch(inactive) to the place Intermediate I. This token value is determined by the firing speed $m7/200$ ($m8/200$). To define the repression level of the processing of Intermediate I to Intermediate II, we use the following formula;

$$\frac{\alpha \times m2}{\beta \times m6 + m2}, \tag{1}$$

which is assigned to the transition T_a. This formula describes the following two functions;

- the firing speed of the transition T_a becomes faster as the amount $m2$ in the place Notch(inactive) increases, and
- the firing speed of the transition T_a becomes slower as the amount $m6$ in the place Delta increases.

Note that the firing speed of the transition T_a can be manipulated by changing the two parameters α and β.

The production rate of Delta is defined by the parameter d at the transition T_b. The forced-expression rate of Delta can be also set to the parameter d_m at the transition T_c.

4 Experimental Results

The large intestine of *Drosophila* embryo occupies a major middle portion of the hindgut, and is subdivided into dorsal and ventral domains (Figure 4 (a)). A one-cell-wide boundary cell strand forms between the dorsal and ventral domains [13] (Figure 4 (a), (b)). Delta is expressed exclusively in the ventral domain, and essenial for the activation of Notch signaling in abutting Delta-negative dorsal cells [14,15,16]. In Delta mutant embryo, in which no Delta protein is produced, boundary cells failed to form (Figure 4 (c)). When Delta protein is expressed throughout large intestine by the GAL4-UAS system, an established method for forced gene expression [17], boundary cell formation was strongly affected. In about 60% of large inestines examined, only a few boundary cells formed randomly (Figure 4 (e)). About 20% of large intestines failed to form boundary cells (Figure 4 (d)). Large intestines with many boundary cell clusters were occasionally found (about 20%, Figure 4 (f)).

These results are summarized as follows:

- Boundary cell strand forms between the ventral and dorsal domains when Delta is expressed only in the ventral domain, i.e., in the case of normal large intestine.

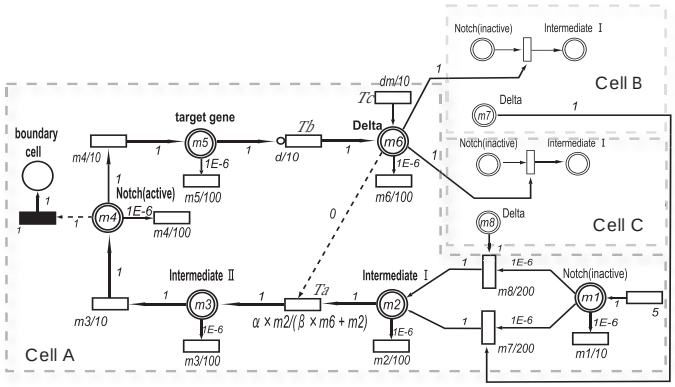


Figure 3. The HFPN model of the Delta-Notch signaling pathway. An HFPN model of complete intracellular circuit of a single Cell A with interactions with adjacent Cells B and C is illustrated. Continuous places represent concentrations of the molecules depicted in Figure 1. Production rates and degradation rates are assigned to the continuous transitions. When the discrete place boundary cell gets token(s), the corresponding cell becomes a boundary cell. Test arcs are used at the reactions where no substances are consumed. Inhibitory arcs are used for modeling repressive activity. The weight *1E-6* which is assigned at some transitions represents 10^{-6}. This means that if the token value of the relevant place becomes over 10^{-6}, the transition begins to fire.

- Boundary cells fail to form in the absence of Delta.
- Forced expression of Delta throughout the large intestine suppresses boundary cell formation, with ectopic induction of a small number of boundary cells.

5 Simulation by Genomic Object Net

Genomic Object Net (GON) is a biosimulation system which is developed based on the hybrid functional Petri net (HFPN) and XML architecture [8].

Genomic Object Net (GON) consists of two tools, GON Assembler and GON Visualizer. GON Assembler allows us to model target biopathways without complicated mathematical formulas, and to perform simulations easily by manipulating parameters directly and smoothly using its GUI [4,9,18]. GON Visualizer was developed based on XML technology [8]. Users can realize visualization of simulation results of biological phenomena by describing it as an XML document, in which CSV files produced by GON Assembler are included

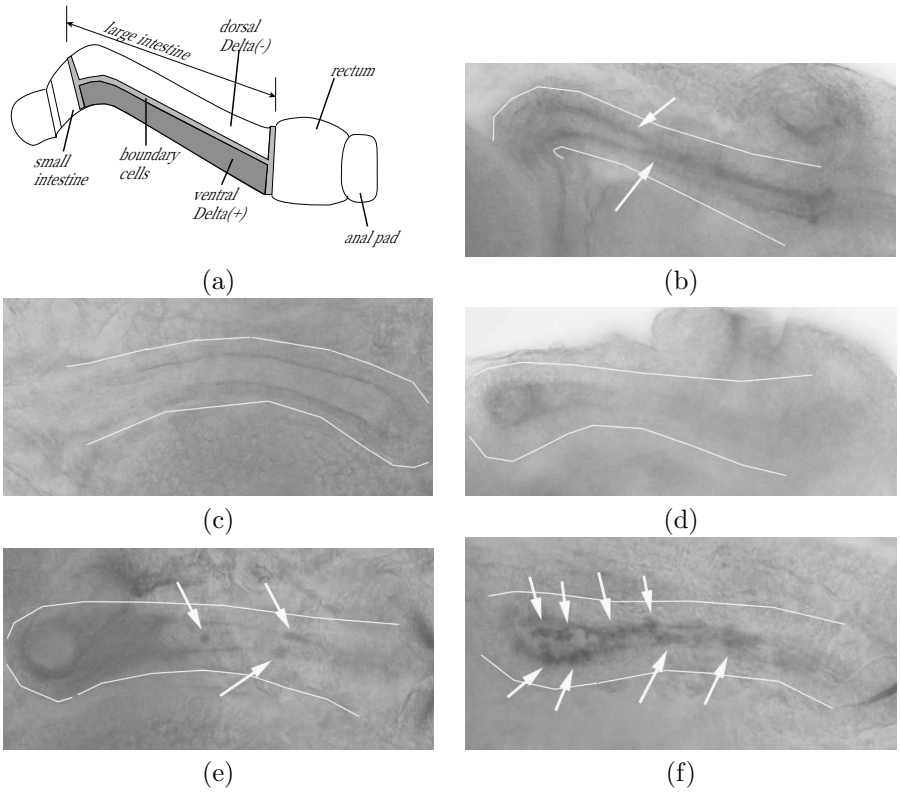


Figure 4. (a): A diagrammatic illustration of the hindgut of *Drosophila* embryo. The large intestine is a major middle portion of the hindgut, and subdivided into the dorsal and ventral domains. A one-cell-wide strand of boundary cells develops between the two domains. (b): Boundary cell strand in the large intestine of a wild-type embryo. Boundary cell strands in the right and left sides of the large intestine are indicated by arrows. Outline of the large intestine is marked with white lines. Staining of boundary cells (in brown color) was performed by use of anti-Crumbs antibody. (c): Boundary cells fail to differentiate in *Delta* mutant embryo. (d), (e), (f): Forced-expression of Delta caused suppression of boundary cell differentiation. In (d), no boundary cells have developed. In (e), a few boundary cells have formed ectopically (arrows). Most of the large intestines examined showed these two patterns. (f) In fewer cases (less than 20% of large intestines examined) many clusters of boundary cells were induced (arrows).

as basic data for visualization. With GON Visualizer, users in biology and medicine can design a personalized visualization for simulation suitable for

the purpose of their studies [8]. By combining these two tools, GON provides an efficient environment for biopathway simulations.

5.1 Simulation model

We carried out simulations of the patterning of boundary cells by using GON. Figure 5 shows the simulation model consisting of 60 cells. Each cell has the HFPN model illustrated in Figure 3. Refer to the website [19] for the full connection model.

For representing cell-to-cell interactions, arcs are drawn from the place Delta of (up to 6) adjacent cells to the transitions between the places Notch(inactive) and Intermediate I. Since the whole HFPN model constructed in this way is very complicated and messy, it is actually difficult to monitor progress of the simulation on GON Assembler.

To address this issue, we wrote an XML document for GON Visualizer which realizes a model of 60 cells (Figure 6 (a), (b), Figure 8 (a)). In this model, the color of each cell can be changed according to the token value in the places which we want to observe.

5.2 Simulation results

Figures 6 presents the simulation results of boundary cell formation. Parameters used in the simulations are summarized in Table 1. We choose parameter values 0.7 and 49 for α and β, respectively. Initial condition for Delta level (d) is: 0 for cells 1-36 (dorsal cells) and 10 for cells 37-60 (ventral cells). This condition represents a prepattern of Delta expression in normal large intestine, in which Delta is expressed only in the ventral cells.

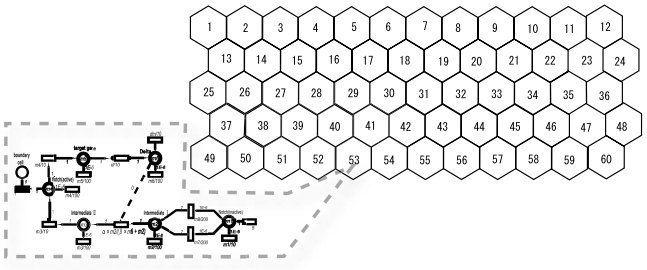


Figure 5. 60 cells model for simulation by GON. Each cell has the HFPN model presented in Figure 3.

Simulation with this condition generated a single strand of boundary cells that are abutting ventral cells (a). The values obtained for Delta ($m6$) and Notch(active) ($m4$) of the cells marked with bold lines in (a) are shown in Figure 7 (a). For the condition of *Delta* mutant, the parameter value of $m8$ was fixed at 0, resulting in no boundary cell formation (b). For the simulation of forced expression of Delta, parameter values 6 and 30 are chosen for d_m.

In the condition of forced expression of Delta at $d_m = 30$, no boundary cells were generated (c), while a few boundary cells were generated ectopically at $d_m = 6$ (d). The values obtained for Delta ($m6$) and Notch(active) ($m4$) of the cells marked with bold lines indicated in (d) are shown in Figure 7 (b). Note that ratios of Delta ($m6$) to Notch(active) ($m4$) of the cells around boundary cells are slightly higher than those of boundary cells in this case. These results correspond well to the experimental results described above (Figures 4), though many boundary cell clusters could not be generated in the present condition.

We also tried a simulation with an initial condition of a uniform Delta level ($d = 3$) for all the cells, in order to represent a situation of lateral inhibition, in which specified cells are singled out from equivalent precursors. When the parameter β was reduced to 5, a regular distribution pattern of specified cells (with a high Delta level) was obtained (Figure 8 (a)), a pattern of which is considered to correspond to the patterning event regulated by lateral inhibition, such as neural cell determination and ciliated cell differentiation in *Xenopus* embryo [1]. The values obtained for Delta ($m8$) and Notch(active) ($m4$) of the cells in the area indicated in Figure 8 (a) are shown in Figure 8 (b).

It should be emphasized that all these patterns were obtained only by changing a few parameter values and initial conditions of a common HFPN model, which is a reasonable approach because all the cells of a multicellular organism are equipped with a common genome.

6 Discussion

Delta activates Notch of adjacent cells, and, at the same time, represses autonomously Notch signal transduction within Delta-positive cells. Activated Notch, in turn, autonomously represses Delta expression. The ambivalent nature of Delta on Notch signaling may lead to rather complicated results when expressed ubiquitously. Forced expression of Delta largely suppressed boundary cell formation, and, at the same time, induces a few boundary cells ectopically. Similar patterns of boundary cell formation were also generated computationally by GON. Obtained ratios of Delta ($m6$) to Notch(active) ($m4$)

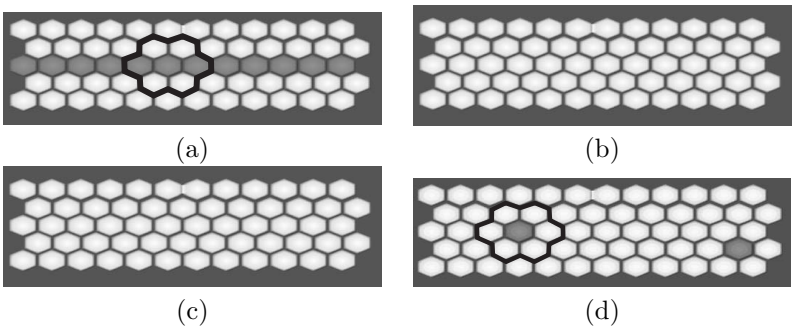


Figure 6. Simulation results of boundary cell formation. Gray cells represent boundary cells. Area marked with bold lines correspond to those illustrated in Figure 7. (a) wild type. (b) *Delta* mutant (realized by removing arc from the transition T_b to the place Delta) (c) and (d) Forced-expression of Delta. Parameter values 30 and 6 were chosen for d_m of (c) and (d), respectively.

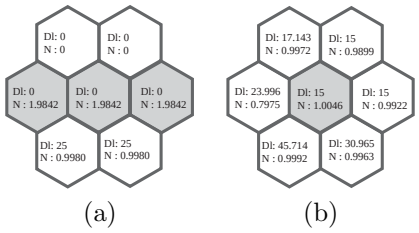
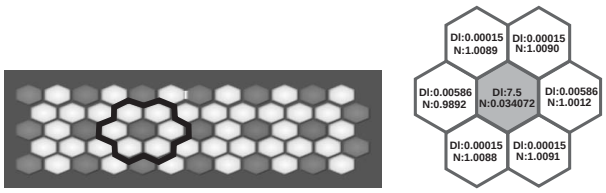


Figure 7. Values of Delta ($m6$) and Notch(active) ($m4$) generated by the simulation in the cells marked with bold lines in Figure 6. Note that ratios of Delta ($m6$) to Notch(active) ($m4$) in cells around a boundary cell are slightly higher than those of the boundary cell.

in cells around boundary cells are slightly higher than those of boundary cells in these cases. Local differences in ratios of the contents of places Delta to Notch(active) occurring among cells is considered to induce boundary cells.

In addition, by reducing parameter value β to 5, with a modification of the initial condition d, GON simulation brought about another type of Notch-dependent patterning, the lateral inhibition. This suggest that two distinct types of Notch-dependent patterning, boundary formation and lateral inhibition, is a consequence of different β values, which represent the susceptibility to autonomous suppressive activity of Delta on Notch signaling.

GON has been demonstrated to be a useful tool for modeling and simulating intracelluar biological phenomena through several examples [5,8,9,18]. In



(a) (b)

Figure 8. Simulation result representing cell specification by lateral inhibition mechanism. (a) Gray cells represent specified cells expressing high level of Delta ($m6$). (b) Obtained values of Delta ($m6$) and Notch(active) ($m4$) in the cells marked with bold line in (a).

Table 1. Parameters in HFPN model of Figure 3 used in the simulation. α and β represent the firing speed of the transition T_a in the formula (1). In the case of boundary cell, different initial production rates of Delta d at the transition T_b are set to dorsal cells:1-36 and ventral cells:37-60, while the same initial value 49 is set to all 60 cells in the case of lateral inhibition. d_m is the forced-expression rate of Delta assigned to the transition T_c.

phenomenon	Figure	(1)		d		d_m
		α	β	cell:1-36	cell:37-60	
boundary cell	7 (a)	0.7	49	0	10	0
	7 (c)	0.7	49	0	10	30
	7 (d)	0.7	49	0	10	6
lateral inhibition	9 (a)	0.7	5	3	3	0

the present study, we tried to model and simulate multicelluar phenomena by using GON, and succeeded in obtaining results corresponding to experimental observations. It is expected that variable multicelluar phenomena can be computationally analyzed by GON based on the technique demonstrated in this paper.

Although GON Assembler has an excellent GUI which allows us to tune up parameters smoothly, it is still difficult to obtain intuitive observations of simulation results, since GON Assembler can present only time-course graph representations. As is demonstrated in this paper, with the support of GON Visualizer, we can realize a more effective and creative environment for biopathway simulations.

We are currently developing a new version of GON Assembler which has the scalability in modeling and simulating more complex biological systems such as the development mechanism of *C. elegans* embryo. For this purpose,

we extended HFPN architechture by allowing more "types" of data (integer, real, boolean, string) with which more complex information such as localization, multicellular process, etc. can be handled smoothly.

Acknowledgments
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