*J. theor. Biol.* (1999) **199**, 243–256
*Article No.* jtbi.1999.0952, available online at http://www.idealibrary.com on IDEAL®

# Isologous Diversification for Robust Development of Cell Society

KUNIHIKO KANEKO*‡ AND TETSUYA YOMO†

**Department of Pure and Applied Sciences, University of Tokyo, Komaba, Meguro-ku, Tokyo 153, Japan and †Department of Biotechnology, Faculty of Engineering, Osaka University 2-1 Suita, Osaka 565, Japan*

(*Received on 5 May 1998, Accepted in revised form on 6 April 1999*)

Isologous diversification, proposed for cell differentiation, is shown to be stable against molecular and other external fluctuations, where amplification of noise-induced slight difference between cells leads to a noise-tolerant society with differentiated cell types. It is a general consequence of interacting cells with biochemical networks and cell divisions, as is confirmed by several model simulations. According to the theory, differentiation proceeds first by loss of synchrony of intracellular oscillations as the number of cells increases. Then the chemical composition of the cells is differentiated. The differentiated compositions become inherited by the next generation, and lead to determined cell types. As a result of successive occurrence of the cell differentiation, the cell society will be composed of different cell types. The whole developmental process is robust not only against molecular fluctuations but also against the removal of a cluster of cells. This robustness is a remarkable feature of isologous diversification, in contrast to the conventional threshold-type mechanism for development. As a testable consequence of the theory, we also discuss interaction-dependent tumor formation and negative correlation between growth speed and chemical diversity.



## 1. Introduction

The developmental process still fascinates many biologists. How can a single fertilized egg give rise to a body consisting of many different cell types and showing distinctive patterns? At early stages of development, a gradient of morphogens in a fertilized egg brings about asymmetric cell divisions, which in turn lead to spatial pattern such as anteroposterior and dorsoventral axes (Alberts *et al.*, 1994). The spatial asymmetries formed by cells then lead to creation of further asymmetry. As development proceeds in constructing more detailed structures, neighbouring similar cells should be able to recognize small differences among them in order to differentiate. For instance, cells in an equivalence group, though their physiological conditions are almost the same, take different developmental paths (Greenwald & Rubin, 1992) as they somehow recognize a small difference in their surroundings.

In general, small differences among cells brought about by their surroundings or by physiological conditions can be amplified if a given threshold of some biochemical is within the range of the difference. That is, if, within one cell, the concentration of a certain chemical (for instance a morphogen) exceeds a threshold value, the corresponding gene is switched on. Conversely, if a neighbouring cell has a bit lower concentration of the biochemical, it turns off the gene.

‡ Author to whom correspondence should be addressed. E-mail: kaneko@cyber.c.u-tokyo.ac.jp.

0022-5193/99/015243 + 14 $30.00/0

Kauffmann, in his Boolean network of genes, has actually demonstrated that such differences in switching of the gene between neighbouring cells lead to cell differentiation (Kauffman, 1969).

However, does this threshold mechanism hold true in all cases of development processes? One of the authors and his colleagues (Ko *et al.*, 1994) showed that *Escherichia coli* arising from a single cell resulted in some distinct cell types upon growth under a homogeneous condition. Although the *E. coli* cells were successively cultivated in a well-stirred liquid culture where spatial bias is minimized, the cells showed different characters and the fraction of each cell type exhibited a complex oscillation. The amplitude of oscillation was higher than expected, compared with the fluctuation of a certain chemical around a threshold. These results imply that the threshold mechanism may not be the main factor for cell differentiation and that cell differentiation is not due to a special mechanism only in higher organisms, but rather is a natural property of cell aggregates, be it simple or not. Moreover, Rubin, in a series of papers has shown that a cell line (N1H3T3) from a mouse transforms to some different types of foci in size under the same condition (Chow *et al.*, 1994; Rubin, 1994a, b). The frequency of the transformation and the types to which the cells are transformed depend on the cell density and the history of the cell culture before the transformation. This implies that some cell differentiations are governed not by a fixed threshold of certain chemicals but rather by dynamical intercellular interaction.

The above-mentioned experimental results challenge the threshold mechanism when it comes to the robustness of development processes. In the traditional threshold mechanism, it is generally believed that genes switch on and off depending on the concentrations of signal molecules. Indeed, several researches have done good justice in showing how cells interact with each other through signals, and how such received signals are amplified to change the state of a cell. However, since each signal involves stochastic error due to molecular fluctuations, the issue then basically concerns how the matured body comes to have a well-developed form with all the accumulated uncertainties involved in the whole development.

Let us assume that there are $M$ genes, each of which is turned on or off in accordance to a signal with the threshold of $N$ molecules. Each on/off pattern of $M$ genes corresponds to a cell type. Here, we note that there exists fluctuation in the order of $\sqrt{N}$ for "$N$" molecules as a result of Brownian motion. This stochasticity is due to a general consequence of probability and thermodynamics, and cannot be avoided. With this fluctuation, the probability that a gene is not switched on or off as designed is $1/\sqrt{N}$. Then the probability that a "correct" cell type is formed as designed is $(1 - 1/\sqrt{N})^M \approx \exp(-M/\sqrt{N})$. This probability is so small (for example, 0.04 for $N = 1000$ and $M = 100$) that the developmental process will scarcely proceed constitutively. Same thing happens when the threshold numbers $N_i$ of signal molecules are not equal, where the probability to have a correct cell type is given by $(1 - 1/\sqrt{N_1})(1 - 1/\sqrt{N_2}) \ldots (1 - 1/\sqrt{N_M}) \approx \exp(-\sum_{j=1}^{M} 1/\sqrt{N_j})$. In this case, the existence of a signal molecule with a small $N_i$ gives a probability too low to allow correct developmental process.§

One might expect that the error in development process would be overcome by the interplay among the genes (Kauffman, 1969). For example, let us assume that a certain cell type is reached starting from several different gene expression patterns. Even though errors occurred and changed the gene expression within the available patterns that give rise to the same cell types, the error would not affect the developmental process of the cell. However, if the error occurred during the differentiation of a cell to another cell type with the threshold of $N$ molecules, the gene expression patterns will then be affected. The fluctuation of $1/\sqrt{N}$ order then exists for each cell differentiation to a new type. Hence, the probability to have a correct set of $k$ cell types is $\exp(-k/\sqrt{N})$.

Still one might argue that the error could be eliminated by proofreading mechanisms existing in a cell, to support the threshold mechanism. However, such proofreading mechanisms will

§If the threshold is not sharp, another source of fluctuations will be introduced. The problem of robustness cannot be resolved with the use of smooth threshold.

only be triggered when a certain condition is satisfied. Since the required condition is also error prone, then another molecular fluctuation sets in. In short, different mechanisms to correct errors in a cell give rise to a chain of fluctuations. Hence, there will always be fluctuations even if we consider all possible fine tunings of the threshold value through evolution.

Each cell differentiation comprising the whole development is inevitably accompanied by stochastic errors, since it is triggered by stochastically fluctuating events like diffusion of a molecule or binding to a certain receptor, which are no other than the so-called signals in most of the researches. Assuming that the embryo is a machine like a parallel processor, then all cell differentiations occurring in the development have to follow a strictly organized course. However, this does not hold true due to the uncertainty of each cell differentiation. Therefore, the whole development cannot proceed in a machine-like manner via only the threshold mechanism.

Previously (Kaneko & Yomo, 1994, 1997), we have presented a simple model for cell differentiation.¶ The model encompasses the basic features of a cell, namely, an intracellular reaction network, cellular interactions through chemicals which serve as signals, and cell divisions. Simulation of the model has shown that cells amplify small intercellular differences through cell–cell interactions, resulting in differentiation into some distinct cell types. The phenotypes of the cells, together with the interactions among them, continue to change until the same cell types and interactions appear recursively through the development.

In this paper, we will first present an overview of the observed events in the simulation of several simple models and clarify the concept of isologous diversification. The concept states that the intracellular reaction network and intercellular interaction bring forth cell differentiations. Here a threshold mechanism is not implemented in advance, but a behaviour like the threshold emerges as an outcome, accompanied with robustness in the development process.

Then, we demonstrate how this isologous diversification leads to such robustness against external noises. In fact, the diversity of cell types as well as their number distribution is stable in spite of the stochastic errors included in the model. Consequently, the development process is expected to be robust even under external noise. Here, each intracellular dynamics can be unstable initially. With the increase in the number of cells, the network of signals brought by cell–cell interactions is selected so that the instability is removed. Robustness of the whole development is a consequence of such dynamical interaction among cells. Note that the concept that interaction is important for developmental robustness is also discussed as the community effect (Gurdon *et al.*, 1993; Monk, 1997) from experimental observations, as well as from theoretical considerations (Newman & Comper, 1990).

The concept of isologous diversification can be applied not only to cell differentiation but also to any systems consisting of replicating units such as the human society and an ecological system. In addition, application of the isologous diversification theory to other scientific fields, such as medical treatment, is discussed.

## 2. Some Basic Features of Cell in the Models

What type of a model is best suited for a cell for investigating cell differentiation? With all the current biochemical knowledge, we can say that one could write down several types of intended model for cell differentiation. Due to the complexity of a cell, there is a tendency of building a complicated model in trying to capture the essence of a cell. However, doing so only makes it difficult for one to extract new concepts, although simulation of the model may produce similar phenomena to those in living cells. Therefore, to avoid such failures, it may be more appropriate to start with a simple model that encompases only the essential factors of living cells. Simple models may not produce all the observed natural

¶There are related studies on the stability in metabolic networks, based on the concept of an attractor (Newman, 1972). Kauffman's model (Kauffman, 1969) also adopts the attractor concept in the genetic network, but it cannot support the stability in the differentiation process as mentioned. Stability of determined cell types, as will be seen at the fourth stage of our scenario, is related to that of an attracting state, but not exactly an attractor. To consider the stability of dynamic developmental *process*, we need some concept beyond that of an attractor, since a cellular state cannot stay at one attractor to differentiate to a new type.

phenomena, but are comprehensive enough to bring us new thoughts on the course of events that have taken place in nature.

To investigate cell differentiation, we have adopted some simple models consisting only of basic features of cells (Kaneko & Yomo, 1997). Some common features are extracted from the results of computer simulations of several models, although, of course, there remain specific phenomena depending on each model and the parameter values adopted. A run-through on the common characteristics brings us essential features and mechanisms in cell differentiation.

The environment of a cell is always taken as one of the essential factors for cell differentiation. If there is spatial bias among the cells, for example, a gradient of an activator protein, the cells may easily undergo differentiation. However, experimental studies on some bacterial cultures (Ko *et al.*, 1994) showed that even in a homogeneous environment, cells can differentiate in due time. In other words, spatial bias is, in fact, not required for cell differentiation. Hence, the cells in our models are grown in a homogeneous environment where there is no spatial bias in chemical concentration. (In fact, by further allowing for spatial bias in our model, we have also confirmed that the cells in our model show the expected cell differentiation.)

Our model consists of intracellular biochemical reaction dynamics, cell–cell interaction through medium, molecular fluctuation, and cell division (see Fig. 1 for schematic diagram). Let us describe each process.

### 2.1. INTERNAL BIOCHEMICAL REACTION NETWORK

The reaction network in a cell consists of numerous biochemicals. A protein activates a certain gene, whose product (e.g. enzymes) catlyses a certain metabolic reaction. The product of metabolic reaction plays the role of an activator to the previous activator protein or to an enzyme in the intermediate reaction. These chain reactions or autocatalytic reactions in a cell (or simply the nature of catalytic reaction with activation or inhibition) constitute a nonlinear reaction network.

To be specific, we choose the concentration $x_i^l(t)$ of the chemical $l$ of the cell $i$ as dynamic

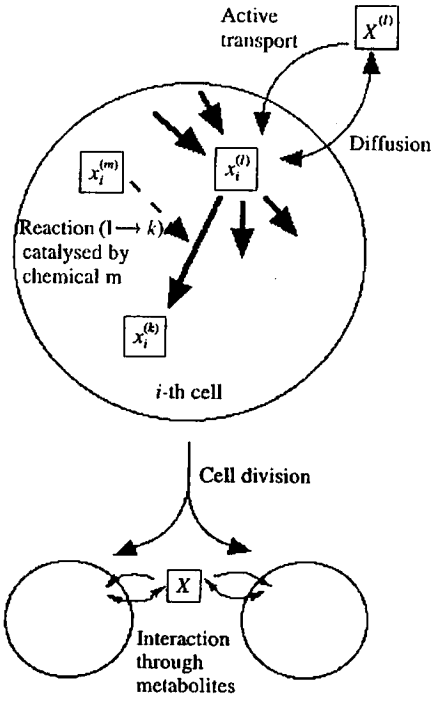


FIG. 1. Schematic representation of our model. See the appendix for the specific equation of each process.

variable, and study its evolution. With the internal reaction dynamics from the chemical $m$ to $l$ catalysed by the chemical $j$, $dx_i^m(t)/dt$ includes the Michaelis–Menten term $e_1 x_i^{(j)}(t) x_i^{(m)}(t)/(a_M + x_i^{(m)}(t))$. The total intracellular dynamics consists of a network of reactions. This network is chosen randomly and fixed throughout the simulation. When the reaction is autocatalytic, $j$ is the same as $m$, in the above term. Such autocatalytic reaction often leads to oscillatory behaviour in chemical concentrations.

As shown by Hess & Boiteux (1971), concentrations of metabolites involved in glycolysis oscillate due to the nonlinear reactions catalysed by some enzymes involved in the reaction network. In general, the biochemical reaction network of a cell is composed of highly complex nonlinear reactions. Hence, we choose such reaction network allowing for oscillatory dynamics.** The concentrations of the biochemicals

** Indeed, the proposed scenario may work, without oscillatory dynamics, as long as some instability is included. Also, in some models, oscillation that existed in initial stage of cells disappears for some cell types determined later.

may be regarded as that of metabolites or expression level of a gene or of a gene network.

### 2.2. INTERACTION AMONG THE CELLS

There are many types of cellular interaction ranging from the diffusion of a morphogen to simple resource competition among cells. However, before trying to identify all cellular interactions, it may be more worthwhile to know the most fundamental type of intercellular interaction. Let us consider some of the simplest organisms like *Anabaena*, a cyanobacterium or *Escherichia coli*. Under ammonia or nitrate deprivation, cells of *Anabaena* differentiate into two types, one specialized for nitrogen fixation, and the other for photosynthesis (Golden *et al.*, (1985). To our knowledge, no reports, to date, except one,†† have suggested any special mechanism that governs its cell differentiation. In the case of *E. coli*, one of the authors (Ko *et al.*, 1994) has shown that *E. coli* cells can differentiate even in a homogeneous environment. Simple as they are, the most probable fundamental factor to be considered is then the interactions among the cells through the biochemicals in the reaction network of the cells.

Our models, therefore, include a simple diffusion process of biochemicals in and out of each cell. By denoting the concentration of the chemical $m$ in the medium by $X^m$, the diffusion term from the medium to the cell $i$ is given just by $D^m(X^m(t) - x_i^m(t))$, while the chemical in the medium is reduced by $-D^m\sum_i(X^m(t) - x_i^m(t))V_{rel}$, where $V_{rel}$ is the ratio of the volume of each cell to that of the medium.

In some models, besides the simple diffusion process, we have also included active transport from the medium to the cell, which keeps the cellular state out of equilibrium. This gives a transport term $X^m \times F$, with some activity $F$, which generally depends on the concentrations of biochemicals in the cell. For example, the activity $F$ is given by sum of chemicals in the cell (see also the appendix). Again, this term leads to global cell–cell interaction through the medium.

### 2.3. CELL DIVISION

To proliferate, a single cell undergoes cell division and is consecutively maintained in the society. In our models, cell divisions occur when the sum of concentrations of some chemicals reaches a given threshold value. In one class of models (adopted in the present paper), the concentration of a "final" product determines the next division.‡‡ It is to be noted that two cells arising from a single cell contain almost identical chemical compositions with a slight deviation (0.1% in most of the simulations). Differentiation occurs among the cells, although a cell in our model divides into nearly identical ones.

Cell division and intracellular dynamics are mutually related. It will be shown below that the intracellular dynamics show oscillation in an ensemble of cells. This oscillation is maintained by cell–cell interaction. If one of the cells divides, the balance that maintained a certain type of oscillation is disturbed, because the new cell introduces new interactions into the system. As a result, the dynamics of the individual cells will continue to change unless cell division is inhibited. Cell division is one of the factors that gives rise to different oscillation dynamics of the cells in a population. In fact, in an "open chaos" system, where the number of variables grows as in a cell culture system, several types of different dynamics appear successively, even if each element alone has simple oscillating patterns (Kaneko, 1994a, b).

### 2.4. MOLECULAR FLUCTUATION

So far we have adopted the model given in the earlier paper (Kaneko & Yomo, 1997), based on the intracellular rate equation of chemical reaction and cell–cell interaction, and cell division. Here, the rate equations of chemical concentrations are obtained by neglecting molecular fluctuation, which is validated if the number of molecules is large enough. Then, "continuous" variables $x_i^m(t)$, concentrations of chemicals, are adopted instead of "integer" numbers of molecules. In reality, the number of molecules for each

†† Intercellular mechanism for pattern formation in *Anabaena* is recently studied experimentally (Yoon & Golden, 1998). It may provide an example of the isologous diversification mechanism, presented here.

‡‡ In another model (see Furusawa & Kaneko, 1998), the sum of all chemical amounts, giving a cell volume, determines the division. The scenario to be presented holds in this case also.

chemical is not necessarily huge, and the number of each signal molecule is typically of the order of 1000 or so (Alberts *et al.*, 1994). Thus, a noise term should be included to take into (thermal) fluctuations arising from finiteness in the number of molecules. Considering fluctuations of $\sqrt{N}$ for the reaction of $N$ molecules, we have added a noise term proportional to $\sqrt{x_i^m(t)}\eta(t)$, with a random force $\eta(t)$, represented by a Langevin equation. The amplitude of the noise is denoted by $\sigma$ (see the appendix for the specific formulation of our model equation).

## 3. Five Stages of Isologous Diversification

Here we present a scenario for the development of cell society, extracted from several simulations (see Fig. 2). Indeed, the scenario, originally extracted from simulations without molecular fluctuations, works completely well up to some noise threshold, as will be discussed in Section 5. The scenario is summarized as follows:

(1) *Synchronous oscillations of the chemicals in the cells.* Only up to a certain number of cells (for example, eight cells) can the dividing cells from

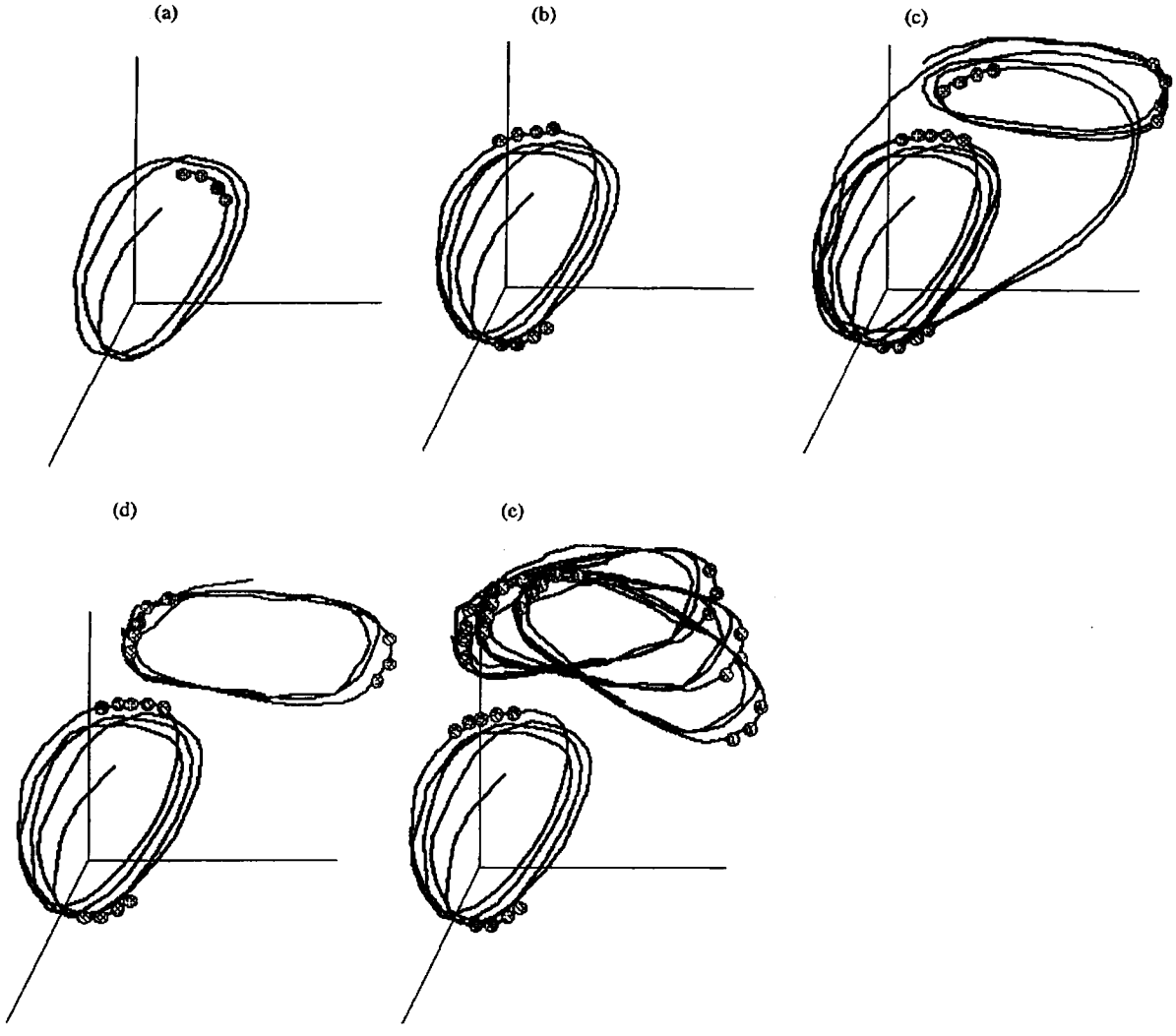


FIG. 2. Schematic representation of the five stages in isologous diversification. Locus indicates the change of chemical concentrations in each cell, plotted in the phase space $x_i^l(t)$, while each circle represents the chemical state of each cell at some instant. (a) Synchronous oscillation (b) clustering in the phases of oscillations (c) differentiation in the composition (d) determination (recursive orbits) (e) hierarchical differentiation. These results are supported by numerical simulations of several models.

a single cell have the same characteristics. Though each cell division is not exactly symmetrical due to the accompanying noise-level of perturbation in the biochemical composition, the phase of oscillations in the concentrations is synchronized. Consequently, cells divide almost at the same time. Such synchronous cell division is also observed with the cells in the embryogenesis of mammals up to eight cells. In our model, the synchronous division is kept until small differences due to molecular fluctuations or slightly unequal distribution of metabolites in each cell division are amplified.

(2) *Clustering in the phases of oscillations.* As slight differences among cells are amplified, the synchronous oscillation breaks and hence, cells start to show different phases of oscillations. Cells split into few clusters where the cells belonging to each cluster are identical in phase. However, this diversification in the phases is not to be mistaken as cell differentiation, since taking the average concentration of biochemicals in time reveals that all the cells are almost identical. Hence, in the second stage of isologous diversification, the cells are only different in their phase of oscillation, brought by the difference in the concentration of biochemicals.

The time course of changes in the concentrations of biochemicals, since it is governed by nonlinear reaction network, is sensitive to slight differences brought by molecular fluctuations or cell division. Once these small differences are amplified, the newly divided cells will produce different phases of oscillations. For instance, if two cells from a single cell have slightly different compositions, the reaction rates of biochemical reaction network are affected by the difference in the phase of metabolic oscillations between the cells, since the reactions involved are autocatalytic. Hence, the phases of metabolic oscillations start to be different between the cells.

Sensitivity of the time course (of chemical concentrations) to tiny perturbation has widely been studied in the field of nonlinear dynamics, in particular in chaos (Lorenz, 1963). Though, according to the nonlinear dynamics theory, perturbations caused by cell division would be amplified so that all the cells would have their own different phases of oscillation, our models show that the cells are not completely diversified in their phases but rather tend to resolve into an ordered clustering.

This clustering of phases is due to cell–cell interaction. In our model, the state with identical cells [i.e. $x_i^m(t) = x_j^m(t)$ for different cells $i$ and $j$] is unstable at the second stage. With this orbital instability, small differences between cells are amplified. However, by forming clusters with different phases, such instability is smeared out. Due to the cell–cell coupling, the dynamics in each group retains stability. Now, small differences in each group are no longer amplified. Chemical observables such as the average concentrations and their oscillatory dynamics are not affected by the stochastic division of the cell, external perturbation from the environment, or molecular fluctuations.

Indeed, the mechanism governing the clustering of the phases is already reported (Kaneko, 1990), where such robustness is mathematically clarified. Small differences among the almost identical cells with nonlinear dynamics are amplified but the phase of oscillations is not completely scattered. Differences in the metabolite concentrations between the clusters are being balanced by the biochemicals secreted from all the cells. The sensitivity to small change is smeared out by this balance. This event in turn leads the cells to stay at a certain phase of oscillation, resulting in the clustering. In other words, it is the transport of biochemicals from one cell to another or cellular interactions (regarded as cell signals) that gives rise to the different clusters in phase.

(3) *Differentiation in metabolite composition.* As the cells with different phases continue to undergo cell division, the average concentrations of the biochemicals over a cell cycle become different between clusters of cells. That is, the composition of biochemicals as well as the rates of catalytic reactions and transport of biochemicals across membrane become different between the clusters.

The temporal difference in the phases leads to the difference in chemical concentrations between cells. The difference of each biochemical activates its transport by a simple diffusion process and makes a small difference in its composition among the cells. The small difference is then amplified through the nonlinear nature of reaction network, leading to clusters of the cells

with different intracellular dynamics. It should be noted that if active transport is applied to the model, the differences in the composition can be observed earlier. That is, a cluster with more biochemicals imports the biochemicals from the medium at a faster rate, thereby hastening the change in the chemical composition between the clusters. Consequently, the cells belonging to each cluster start to possess different average composition over their cell cycle.

The composition of biochemicals of each cell is not an inherent property, since the intracellular dynamics governed by the nonlinear reaction network, generally, if not in all cases, can vary in time. Hence, the composition of a newly divided cell can be different from that of the parent cell. Therefore, each newly divided cell has a different rate of biochemical reactions depending on the composition. In short, cells are at the intermediate stage of the differentiation process.

(4) *Determination of the differentiated cells.* As the cells continuously undergo cell division further, they start to have their own inherent composition that is preserved by the next generation of cells. That is, the cells come to a stage where the reaction dynamics and the chemical composition are not much influenced by the environment and other cells. Hence, the biochemical properties of a cell are inherited to its progeny, or in other words, the properties of differentiated cells are stable, fixed or determined over the generations. This is the fourth stage of isologous diversification.

The balance between the clusetrs, each having its own compositions in the third stage, is attained through cellular interactions in the same way as in the dynamical clustering of the oscillation phase at the second stage. Although the metabolite composition of the cells tends to change over the generations, this tendency is compensated by the intake of biochemicals secreted from the cells belonging to the other clusters. Therefore, this cellular interaction through the biochemicals between the different clusters allows the recurrence in the events of the change in concentrations of the biochemicals over the generations, leading to the determination of the features of the differentiated cells.

As soon as the features of the cells are inheritable, a cell lineage map can be drawn, to show where the cell types branch out from their origin. As shown in Fig. 3, emergence of certain cell types at different branches is observed in the model, similarly in the development process in nature (Alberts *et al.*, 1994; Kenyon, 1985). In Fig. 3, cells of a certain phenotype (e.g. given by "dark blue") arise in isolation individually at different points in a lineage. Therefore, not only the history of each cell lineage accounts for the maintenance of different cell types over the generations. Instead, the global interaction among cells is important. In short, the emergence of certain cell types at different branches is but one of the features of the interaction-driven society.

The obtained cell lineage is not changed even if some fluctuations are included in chemical reaction process and at the cell division process. If the noise term mentioned in Section 2 is applied, the same cell types and the same differentiation process are observed up to a certain noise strength, as will be discussed in Section 5. The cellular states given by chemical compositions are dynamically selected throughout the cell–cell interaction, which cancels out the instability in the intracellular dynamics of each cell. Hence, the determined states and the number distribution of cell types are robust against perturbations due to molecular fluctuations.

(5) *Hierarchical organization of cell types.* By means of global cellular interaction among the clusters of different cell types, the cellular phenotypes are stable at stage 4 of the isologous diversification. However, as the cells continuously proliferate, cellular interaction within each cluster can result in further differentiation of the cells. Subgroups, undergoing the cycle of stages 1–4, are formed within a cluster. This formation of subgroups within clusters is not to be mistaken as disturbance of the stability of the clusters that was attained in stage 4. The difference in the chemical composition among the subgroups is smaller than the difference between the clusters. Moreover, the average composition over the cells in each cluster hardly changes. The interaction between the clusters maintains the stability that was attained in stage 4. In short, the subdivision of each cluster occurs with such small changes that the interaction among the clusters is maintained to give rise to the stability of the clusters in future. Various levels of differences among and

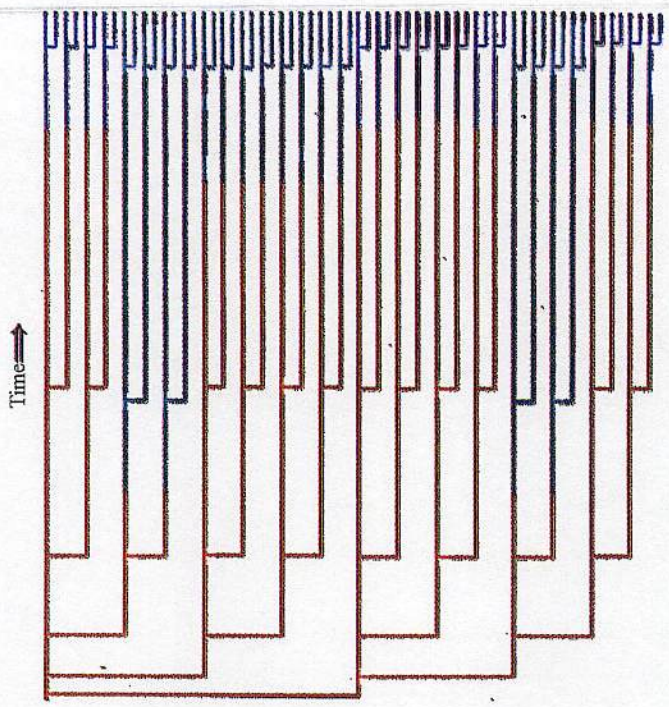


FIG. 3. Cell lineage diagram obtained from a simulation. The colours correspond to the cell's character defined as the average chemical composition of the cell. The red colour corresponds to undifferentiated type (i.e. the particular composition of this cell type may change from division to division). The other colours correspond to determined cell types with different chemical compositions, that do not change after the cell division.

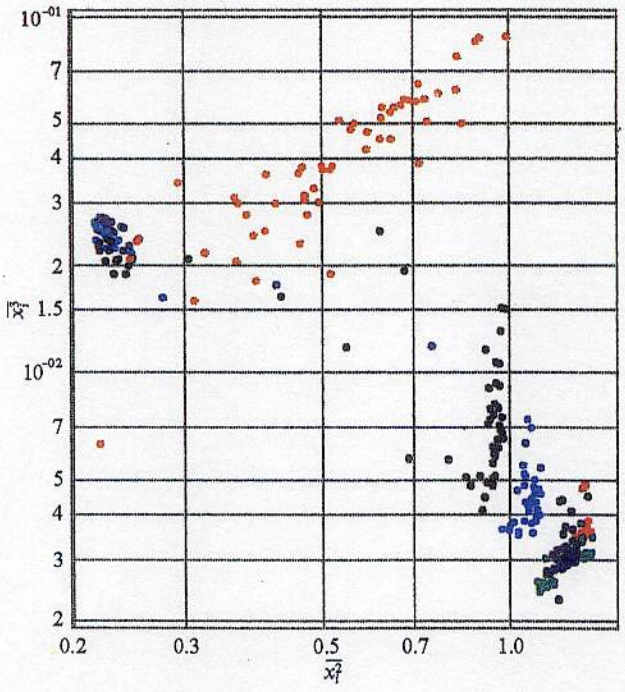


FIG. 5. The average concentrations of $[x_i^2(t), x_i^3(t)]$, at the time step 2000. Plotted are the temporal averages, taken from the latest division to the time step 2000. The noise amplitudes $\sigma$ are 0.0001 (red), 0.0003 (green), 0.001 (blue), 0.005 (light blue), 0.02 (brown) and 0.05 (orange). For each colour, there are a number of points corresponding to each cell existing at the time step 2000 (64 up to $\sigma = 0.005$, 62 for $\sigma = 0.02$, 53 for $\sigma = 0.05$), although for $\sigma \leq 0.001$, some of the points are overlaid and may be invisible since the plotted concentrations of the cells are rather close to each other.

within clusters lead to a hierarchical organization of cell types (see also Kaneko & Furusawa, 1998).

### 4. Level of Differentiation

The hierarchical differentiation of the cell types relates various levels of differentiation. In developmental biology, transplant experiments give information on the level of differentiation and the timing of determination. To know how stable the differentiated features of our model cells are against changes in the environment or in cellular interaction, some of the differentiated cells are mixed and cultivated. This artificial transplant experiment on the model has shown (Kaneko & Yomo, 1997) that a cell tends to keep its differentiated state after mixing with other types of cells. The simpler composition a differentiated cell has, the more the cell tends to keep its own features. In other words, features of the cells having simpler chemical compositions are highly determined. From these computed results of the transplant experiment, we predict that the strength of the determination is negatively correlated to the number of biochemical species in a cell. This prediction can be tested experimentally with biological cells.

Why is a cell with a simpler composition of metabolites highly determined in transplant experiment? Since cells with simpler composition have biased concentrations to fewer biochemical species, they have a higher concentration of each of the biochemicals. As the concentration of each biochemical in the cells is high, influence by interactions with the surrounding cells is weaker and is not sufficient to change the state of their metabolic activity significantly. Hence, simpler composition gives robustness to the cell type.

An extreme example of the above relationship is what could be called a cancer cell in our model simulation (Kaneko & Yomo, 1997). The cancer cells we have found have a very simple composition and grow faster than the other cells, by excluding them from the system. This antisocial feature is apparently due to the simplicity of its metabolic composition. To reverse the cancer cells to a normal state in our model, the diversity in the metabolic composition should be recovered. Indeed, if the cancer cells in the model are mixed with the undifferentiated cells containing various biochemicals, most of them regain a diverse biochemical composition and revert to a normal state. In similar way, by introducing cytosol from undifferentiated cells or egg, into some human cancer cells, through liposomes, their malignancy may be cured.

### 5. Robustness of Developmental Process

In our model, the robustness of the cell society is shown in different stages of developmental process. When the system is perturbed externally by changing the concentrations of the metabolites, still a similar developmental path is taken by the cells in the society. As a result, similar cell lineages are obtained.

To demonstrate this robustness, we have introduced noise in our simulations to represent the molecular fluctuation (given by the Langevin equation in Section 2). In Fig. 4, we have plotted the growth of total cell numbers with time. Up to the noise strength $\sigma_{thr}$, the growth curve is not modified.

To check the robustness of the biochemical compositions, we have plotted the average

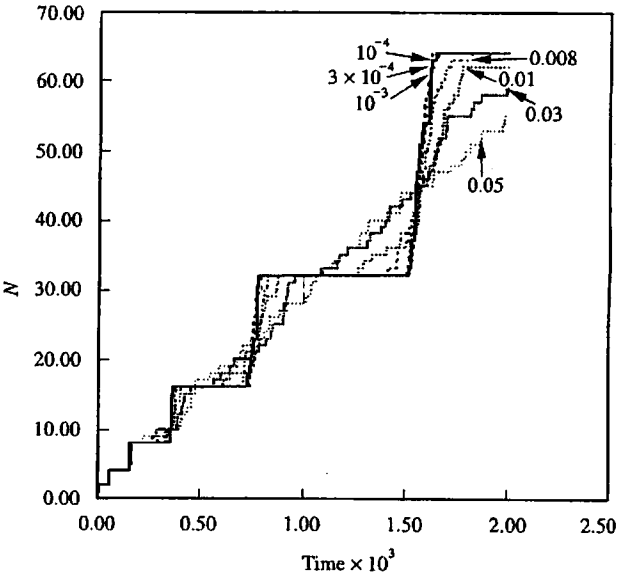


FIG. 4. Increase of the number of cells with time. The time courses for $\sigma = 0.0001$, 0.0003, 0.001, 0.008, 0.01, 0.03 and 0.05 are plotted. For Figs 4 and 5, simulation is carried out by adopting a model given in the appendix with the network given in Kaneko & Yomo (1997), with $p = 10$, $e_1 = 1$, $V = 1000$, $\gamma = 0.2$, and $D = 0.02$, while the flux for $X^0$ is given by the term $D_{out}(S - X^0)$ with $D_{out} = 0.005$ and $S = 40$.

concentrations of two chemicals $[x^2(i), x^3(i)]$ in Fig. 5, at time step 2000. Plotted are the temporal average from the latest division to the time step 2000, when 64 cells split into two distinct clusters with different biochemical compositions, in a simulation without molecular fluctuations. Each point corresponds to each cell existing at time step 2000, while some of the points are overlaid. As shown in the figure, the formation of two distinct clusters is invariant, while their biochemical compositions are hardly modified, as long as the noise strength $\sigma$ is less than $\sigma_{thr} \approx 0.008$. The number distribution of cells at the two clusters lies within the range from $25 \pm 1$ to $39 \mp 1$, independent of the noise strength less than $\sigma_{thr}$.

As discussed earlier, the final cell types are dynamically selected by cell–cell interaction, overcoming the instability brought about by the intracellular dynamics. If the chemical composition or the number of each cell type deviated from that of the selected cell society, the instability would reappear, which would enforce the system to revert to the original composition and distribution. Hence, robustness against molecular fluctuations is a logical consequence of our scenario.

It may also be interesting to study how the differentiation pattern is destroyed when the molecular fluctuation is too large. As shown in Fig. 5 the two types start to merge at $\sigma \approx \sigma_{thr}$, and for larger $\sigma$, no distinct types are observed any more. In this case, the biochemical characteristics of each cell are continuously distributed as in Fig. 5. Hence, cells divide continuously, instead of the stepwise increase of cell numbers that is observed for $\sigma < \sigma_{thr}$. (see Fig. 4).

If the cells cannot form different types exploiting different chemical "niches", the competition between cells for resources is higher. In fact, with the increase of noise amplitude $\sigma > \sigma_{thr}$, the cell replication is suppressed. With the further increase of $\sigma$, some cells lose their chemical activity, and come to a "dead" state. Indeed, in a simulation with $\sigma > 0.06$, a certain fraction of cells loses the ability to grow. For such cells, the amount of chemicals starts to decrease towards zero with time. In other words, if the noise becomes too large, a developmental process with the increase in the cell number no longer occurs.

From the threshold noise $\sigma_{thr}$, it may be possible to estimate minimal number of molecules in a cell required for a robust development process. Note that the concentration variable $x_i^m(t)$ is the number of molecule $N_i^m(t)$ divided by the cell volume $V$ $[x_i^m(t) = N_i^m(t)/V]$. Assuming a fluctuation of the order of $\sqrt{N}$ for a system with $N$ molecules, the noise strength $\sigma$ for the concentration equation (see the appendix) is estimated as $\sqrt{1/V}$. Then the number of molecules necessary to have robustness is given by $N = Vx$ $x/\sigma_{thr}^2$. In our simulation, the concentration $x$ for typical biochemicals is of the order of 0.01–0.1. Hence, the threshold number of molecules in our model is estimated as 100–1000. Of course, we have to admit that this is a rough estimate and that the present model is too simple to claim the number as a value comparable to the number of signal molecules in a real cell. Still, it should be stressed that a huge number of molecules is not required to have robustness, according to our scenario. Here, robust development is possible even with the number of molecules of the order of $10^3$, which is consistent with observations in molecular biology.

Our cell society is not just robust against molecular fluctuations. It is even robust against "macroscopic" perturbations, for example, the removal of some cells. Here, the relevance of cellular interactions to robustness is again confirmed. For example, in some of the simulations, we observed the appearance of stem cells (Furusawa & Kaneko, 1998) that produce various cell types. The rate of differentiation from a stem cell to other cell types is found to be dependent on the cell-type distribution. When the number of cells of a certain type is decreased, the stem cells increase the rate of producing the reduced cell type. As a result, the ratio of the cell type in question in the society is restored, thereby recovering the original state of the society. Thus, the cellular interactions do not only trigger differentiation but they also make a society with diversified cell types robust.

Even though the model disregards space as a factor, a society of diversified cell types exists. It indicates that spatial bias, like a morphogen gradient, is not necessary for cell differentiation. It is to be noted that this statement does not deny

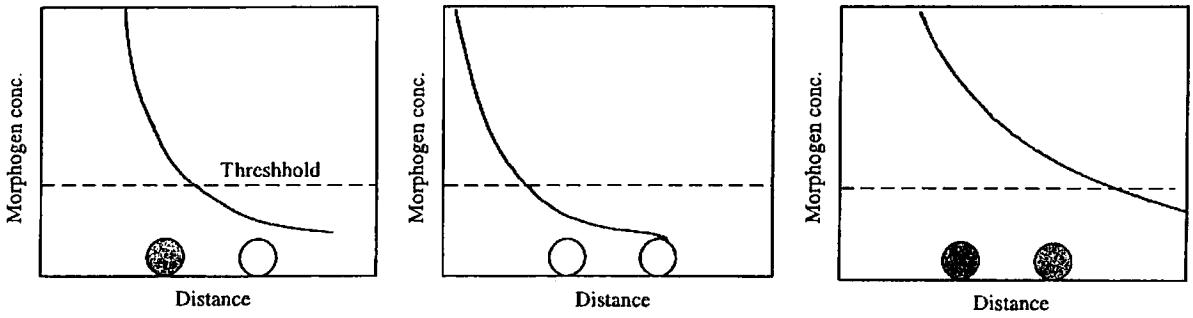


FIG. 6. Instability of the differentiation due to the threshold mechanism. The curved line indicates the morphogen concentration. Cells, if located at the site where the morphogen concentration is above the threshold, differentiate into a certain type (shaded circle), otherwise they keep their original type (white circle). The differentiation succeeds only if the morphogen concentration crosses the threshold at a position between the two cells, as shown on the left.

that in the developmental process, the gradients of morphogens are imperative for constructing the body in space (Nellen *et al.*, 1996). Under a certain threshold of the morphogen concentration shown in Fig. 6, cells differentiate. If the concentration difference of the morphogen between two adjacent cells exceeds the threshold, the cells differentiate. However, if a differentiation process would simply rely on gradients created and maintained by a diffusion process, a developmental process with successive differentiations would not succeed because the diffusion of a small number of molecules is stochastic. Since the diffusion process itself is stochastic, the gradient changes its absolute value so that two adjacent cells may come to have the same features, and fail to be differentiated into two types. Therefore, the threshold mechanism based on the gradient cannot by itself overcome the stochastic effect of diffusion, regarding cell differentiation.

On the other hand, in our isologous diversification, there is no uncertainty in cell differentiation process. Even though the gradient of morphogen around the adjacent cells is perturbed, the concentration difference is amplified and gives rise to cell differentiation. In addition, some experimental studies on equivalent groups of cells proved that cellular interaction is necessary for cell differentiation (Greenwald & Rubin, 1982), as proposed in the isologous diversification theory.

In the same way as each of the cell differentiations, the final ("adult") cell-type distribution is reproducible. If the development process works like a programmed machine, then the signals and switches that trigger each cell-differentiation event must work as programmed. Nevertheless, since only a small number of biochemical molecules trigger each cell differentiation, stochastic errors due to molecular fluctuation are inevitable. Hence, the machine-like mechanism for development cannot cope with the successively and parallelly occurring errors in each cell differentiation. In our model, even without a "fixed" mechanism for controlling cell differentiation, the system shows a reproducible distribution in cell types during the development. Moreover, the transplant experiments show that the whole development is robust against any perturbation such as removing and adding some cells. The robustness of our model cells indicates that the embryo is not necessarily a programmed machine that requires strict control. Rather, it has a certain flexibility to develop a precise cell-type distribution.

Another question here is why the whole development is robust, although each cell differentiation in our model is triggered by amplification of small difference (by stochastic errors). As explained in Section 3, cells have a tendency to change their phenotype until a balance is achieved between the tendency and the global interaction between the cells. Once the balance is established, the cell-type distribution is robust. In other words, the distribution of cell types is protected from perturbations, and is maintained over a certain period. Thus, developmental process selects the global interaction that ensures a reproducible cell-type distribution. Moreover, this self-induced robustness can explain why the development into an elaborate body proceeds coherently even under fluctuating conditions.

### 6. Generality of Isologous Diversification

In the model presented, the isologous diversification occurs even in cells with simple metabolic or genetic networks that interact with each other through the diffusion of biochemicals. This does not deny that more complicated machinery is involved in cell differentiation. Rather, our theory states that cell differentiation is a consequence of dynamic instability due to the metabolic and/or genetic network in a cell, and inevitable cellular interactions no matter what complicated processes are involved. Moreover, since isologous diversification does not require more than what a single cell organism has, it renders some knowledge about the emergence of multi-cellular organisms with differentiated cell types. We also stress that robustness in development is a natural consequence of dynamical instability and cell–cell interaction. For this robustness neither a large number of signalling molecules nor a highly tuned machinery is necessary.

Although chemical compositions are differentiated by cells in our model, one may still wonder if they are really differentiated at a functional level, since any specific functional role of each chemical process is not prescribed in our model. It is to be noted that this paper has not attempted to explain how each biological function develops from undifferentiated cells. Isologous diversification deals with the issue of understanding how almost identical cells are differentiated under stochastic uncertainty. It is necessary, but may not be sufficient to have functional differentiation. However, isologous diversification can be extended to functional differentiation, because the resultant differences in biochemical composition among cells may be amplified by the gene network, hereby leading to new function for each cell. Therefore, from this point of view§§, isologous diversification may contribute to the development of a biological function. To answer the questions as to how and to what extent present organisms utilize the isologous differentiation, however, evolutionary process together with the present differentiation has to be further studied [see Kaneko & Yomo (1999) for this direction].

Due to the simplicity of isologous diversification, it can be applied not only to a cell society but to any society, universally. For instance, in the ant or in the human society, with internal complex dynamics interactions between the individuals, the proposed isologous diversification theory may explain the origin of hierarchical structure of the society. There, in spite of inevitable, internal and external fluctuations to each individual, robust development is generally observed, as, for example, in the development of a colony from a single queen ant. The proposed logic for robustness in our scenario may explain the robustness of any society in general. In short, isologous diversification is applicable to any system with internal complexity of entities which grow and interact with each other.

We would like to thank Roeland Merks and Elizabeth P. Ko-Mitamura for critical reading of the manuscript, and C. Furusawa for stimulating discussion. This research was supported by Grant-in-Aids for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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§§To discuss the problem of function, we need to consider seriously what it really means. Function should be neither chemical composition nor some shape of cell, but some relationship among cells, or tissues, in the context of the whole organism. When some chemical, mechanical, or other action of one unit (cell) is used by other units (cells), for the survival of the whole organism, one tends to assign a function to the action.

In our model, chemical reaction process of one cell-type leads to a specific chemical composition different from the other type. This specialized chemical composition, diffusing out from the cell, works as a boundary condition for the other cells, and stabilizes the chemical state of the other cell-type. To put it the other way around, the specialized composition of one cell-type is used by other cell-types. With this stabilization, cells continue further robust developmental processes, as demonstrated in the present paper. In this regard — formation of mutual relationship for the development of the whole organisms — it may be possible to assign some (primitive) function to chemical reaction or chemical reaction network in our model.

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## APPENDIX

### Our Model Equation

Here we give the explicit model equations used in the simulations in Section 5. The dynamics of the $l$-th biochemical concentration $x_i^{(l)}(t)$, in cell $i$ consists of an internal reaction term *React*, an active transport *Transp*, a diffusion term *Diff*, and molecular fluctuations *Fluct*, as

$$dx_i^{(l)}(t)/dt = React_i^{(l)}(t) + Transp_i^{(l)}(t) + Diff_i^{(l)}(t) + Fluct_i^{(l)}(t). \quad (A.1)$$

In this paper, the reaction term *React* consists of autocatalytic reaction network with a set of reaction paths from $j$ to $l$, written as

$$\begin{aligned} React_i^{(l)}(t) &= S(l)e_0 x_i^{(0)}(t)x_i^{(l)}(t) \\ &+ \sum_j Con(l,j)e_1 x_i^{(j)}(t)x_i^{(l)}(t)/(1 + x_i^{(l)}(t)/x_M) \\ &- \sum_j Con(j,l)e_1 x_i^{(l)}(t)x_i^{(j)}(t)/(1 + x_i^{(j)}(t)/x_M) \\ &- \gamma P(l)x_i^{(l)}(t), \end{aligned} \quad (A.2)$$

where $Con(l,j) = 1$ if there is a reaction path from the chemical $j$ to $l$, and $Con(l,j) = 0$ otherwise. Here we assume that there is a source chemical 0, from which other chemicals are formed by the term $S(l)e_0 x_i^{(0)}(t)x_i^{(l)}(t)$. There is a flow to a product required for cell division, given by the term $\gamma P(l)x_i^{(l)}(t)$. Again, $S(l)$ or $P(l)$ is 1 if such a path exists and 0 otherwise. [The equation form for $x_i^{(0)}(t)$ is obtained straightforwardly.]

With the flow at the rate of $\gamma P(l)x_i^{(l)}(t)$, the product initiating cell division is accumulated. Hence, cell $i$ divides if the condition $\int dt\, \gamma \sum_l P(l)x_i^{(l)}(t) > Threshold$ is satisfied. After the division, the two cells have the same chemical composition as the mother cell $i$, with some tiny fluctuation [0.1% for each chemical $x_i^{(l)}(t)$].

The transport term expresses active transport from the medium to each cell. The rate of transport in general depends on intracellular chemical concentrations. As a simple model, we choose that the activity is given by $\sum_{k=1} x_i^{(k)}(t)$. Then, the term is given by

$$Transp_i^{(m)}(t) = p\left(\sum_{l=1} x_i^{(l)}(t)\right) X^{(m)}(t), \quad (A.3)$$

where $X^{(m)}(t)$ denotes concentration of chemical $m$ in the medium. Apart from the active

transport, chemicals diffuse through the membrane as

$$Diff_i^{(m)}(t) = D(X^{(m)}(t) - x_i^{(m)}). \quad \text{(A.4)}$$

Since the active transport and diffusion processes are just the transportation of chemicals to and from the medium, equations for the chemicals $X^{(m)}(t)$ in the medium are equal to the sum of $Transp_i^{(m)}(t)$ and $Diff_i^{(m)}(t)$ over cells $i$ (divided by the volume ratio of the medium to a cell). [For the source chemical $X_i^0(t)$, we assume a flux from the outside, so that the consumed resources are supplied.]

So far we have adopted the model given in the earlier paper (Kaneko & Yomo, 1997) in details. In the present paper, we add a molecular fluctuation term, given by the Langevin equation

$$Fluct_i^{(m)}(t) = \eta_i^{(m)}(t)\sqrt{x_i^{(m)}(t)} \quad \text{(A.5)}$$

with Gaussian white noise satisfying $\langle \eta_i^{(m)}(t) \eta_i^{(m)}(t') \rangle = \sigma^2 \delta(t - t')$.

Note that the model choice in this paper is just a specific example. In other models such as the model given in Furusawa & Kaneko (1998), the same scenario for the developmental process is confirmed.