Biological robustness is a principle that may shed light on system-level characteristics of biological systems. One intriguing aspect of the concept of biological robustness is the possible existence of intrinsic trade-offs among robustness, fragility, performance, and so on. At the same time, whether such trade-offs hold regardless of the situation or hold only under specific conditions warrants careful investigation. In this paper, we re-assess this concept and argue that biological robustness may hold only when a system is sufficiently optimized and that it may not be conserved when there is room for optimization in its design. Several testable predictions and implications for cell culture experiments are presented.

**Introduction**

It has been claimed that trade-offs exist between robustness, fragility, performance, and resource demands in biological and engineering systems (Csete and Doyle, 2002; Kitano, 2004, 2007). Determination of the conditions in which this conjecture would hold is of great interest for systems theory in biology. For example, systems that are optimized to be robust against certain perturbations are often extremely fragile against unexpected perturbations. This trade-off is also known as the ‘robust yet fragile’ nature of highly optimized systems (Csete and Doyle, 2002). Principles such as the Bode integral formula (Bode, 1945) and the summation theorem in metabolic control analysis (Fell, 1997) underscore this trade-off in certain conditions. Although such theorems provide a basis for understanding robustness trade-offs, their applications are limited to specific aspects of the system. The Bode integral formula applies specifically to conservation of the sensitivities of negative feedback circuits on a frequency axis, and the summation theorem assumes linearity for minor perturbations. In addition, real systems are likely to exhibit more complex robustness-fragility trade-offs because of the involvement of component failures and other aspects not taken into account for these theorems (see Supplementary information). Such trade-offs may thus hold only when design and implementation are sufficiently optimized. This means that the system can be made more robust without undermining other features (see Box 1). It should be noted that the system can be optimized by redesign and reimplementation of engineering systems. In biological systems, an evolutionary selection is required for such design optimizations. Although qualitative observations exist for this trade-off, quantitative experimental verification of this trade-off has not been conducted.

In contrast, the trade-off between robustness and performance is more tractable, and several experimental and computational reports discussing such a trade-off have been published (Ibarra et al., 2002; Stelling et al., 2002; Fischer and Sauer, 2005; Andersson, 2006). In short, the trade-off dictates that high-performance systems are often more fragile than systems with suboptimal performance. Interestingly, there are studies reporting suboptimal metabolism performance in *Bacillus subtilis* and *Escherichia coli* (Stelling et al., 2002; Fischer and Sauer, 2005). If the trade-off holds, metabolic performance has to be kept suboptimal to ensure a certain level of robustness against environmental perturbations.

As such studies observe cultured microorganisms and cells, changes in performance and robustness can be attributed to either of the two scenarios: emergence and rebalancing. The emergence scenario assumes that random mutation gives rise to a genetic subtype that fits the perturbed environment better and that this subtype quickly proliferates in culture. The rebalancing scenario assumes that a specific mutant strain that fits the environment better may already exist in a heterogeneous population even before perturbations are imposed, and that this strain proliferates faster than other strains under the perturbed environment.

It is important to clearly define robustness and adaptation through evolutionary selection. Here, ‘robustness’ means an individual organism’s capability of tolerating external and internal perturbations, such as environmental fluctuations, the addition of drugs, and mutations. Robustness—performance trade-off means that, when two individuals are compared, one is found to be more robust than the other but is outperformed by the other; thus, no individual can be more robust and at the same time exhibit higher performance than others. In general, organisms can be ‘optimized’ or ‘adapted’ to a certain condition by evolutionary selection; thus, they can be more robust against perturbations implicated in the
selection pressure or can have higher performance than preselected individuals. Thus, if the robustness–performance trade-off holds, descendents of organisms can be more robust than their ancestors when they are adapted for perturbations imposed during evolutionary selection, but they may be outperformed by their ancestors or by other individuals adapted for other conditions in which performance is favored. By the same token, the descendents of organisms can outperform their ancestors when selection pressure favors high-performing individuals, but may be less robust than their ancestors and other individuals evolved under conditions that favor more robust individuals.

In this paper, we examine the idea that such trade-offs appear only when the system is sufficiently optimized, and thus may not be observed when systems are yet to be fully optimized. This implies that there should be cases in which descendents of organisms can be more robust and perform as well as or better than their ancestors, which is not possible if robustness–performance trade-off holds universally.

**A primer on portfolio selection**

In this article, the portfolio selection concept used in modern investment theory is introduced to explain robustness–performance trade-offs. Portfolio selection is an idea to combine various investment options to minimize risk while attaining the desired return on investment (Markowitz, 1991). In the modern portfolio theory used in investment practice, it is well understood that there is trade-off between risk (uncertainty of the return shown by s.d.) and performance (expected return). High-yield financial products generally have higher risk, and modest-yield products have lower risk. Risk in this context refers to the s.d. of the asset price. Performance is measured by the expected percentage of return. Any investment item (asset) can be mapped on a yield–risk space.

As investors generally invest in multiple financial assets with different expected yields and risks, the question is how to find the optimal mix of assets with a desirable yield and acceptable risk. The concept of efficient frontier needs to be introduced here. The efficient frontier is a set of points that represent an optimal combination of assets (mostly securities in a financial context) that maximizes the return for any given level of s.d. Any point not on the efficient frontier represents a portfolio that is inferior to a portfolio on the efficient frontier, either because it generates less return at the same level of risk or is exposed to higher risk at the same expected level of return. In Figure 1A, Portfolio X is inferior to both Portfolios Y and Z. Portfolio Y has a higher expected return than X at the same level of risk, and Z has lower risk than X with the same expected return. Portfolio X can be reorganized to reach the efficient frontier. Thus, theoretically, any portfolio not on the efficient frontier can improve its yield without increasing risk, or reduce risk without undermining the expected yield. However, on the efficient frontier, any change in yield affects risk and vice versa. Trade-off between risk and yield takes place on an optimal portfolio that is on the efficient frontier. A similar trade-off concept is also investigated in the context of multiobjective optimization as the Pareto efficiency, originally proposed by Pareto (1935). For a Pareto-efficient solution, no individual parameter can be improved without undermining another parameter. A set of Pareto-efficient solutions constitute a Pareto surface, also called a Pareto frontier.

An indifference curve projects valuation criteria on the yield–risk space. It has graded utility levels depending on...
whether the desired portfolio is selected on the basis of risk preference. A risk-aversion indifference curve represents a portfolio chosen to maximize the expected return but avoids risk (Figure 2A). A risk-neutral indifference curve is used when only the expected return is considered (Figure 2B). A risk-preference indifference curve is used when higher risk is preferred for an equal expected return (Figure 2C). Obviously, the risk-preference indifference curve would be an odd choice for an investment situation. Thus, the risk-aversion indifference curve is used in general.

Genetic portfolio selection: translating investment theory into biology

Portfolio selection, which seems remote from biology, can be applied to understand the evolution of microorganisms and cells in specific conditions. Thus, it may help us understand robustness–performance trade-offs.

Each organism or cell can be mapped onto the yield–risk space. A position of the yield–risk space that characterizes the biological entity X can be called ‘a projected position of X.’ Yield is an expected performance, such as reproduction speed or biomass production rate. Risk (equivalent to fragility) is the degree to which a growth rate or biomass production rate is affected by perturbations. In general, it can be represented by s.d. and calculated by assuming possible perturbations, their probabilities of occurrence, and expected yield under each perturbation (Box 2). These indexes can be measured by tracing behaviors of individual cells and their biomass production or lineage for reproductive efficiency under various conditions. Alternatively, they can be measured by the growth of the population under various perturbations in which the population can be kept monoclonal. In this case, the distribution of projected positions of a certain cell or organism
for its wild type and various mutants on the yield–risk space is contained by the efficient frontier (Figure 3). Changes in the distribution of projected positions in the yield–risk space for randomly sampled cells will test the conjecture that the robustness–performance trade-off holds only at the efficient frontier.

Next, we consider the cases in which such a trade-off holds in a population of microorganisms and cells. Analysis at the population level is biologically important because cell cultures that have a substantial level of heterogeneity are often used in biological experiments. In addition, certain tumors are known to be composed of heterogeneous cancer cells. Furthermore, populations of organisms and cells are used to measure growth rate and how organisms respond to environmental changes in the context of the study of general biology and in drug screening.

Growth rate (yield or performance) is generally measured by the size of a colony, by numbers of cells, or by other means that reflect the number of cells in the population. Risk is an s.d. of growth rate under various possible perturbations. Experimentally, it can be measured by repeated perturbation experiments. In a heterogeneous cell culture, the projected position of the cell culture in the yield–risk space is determined by the population composition. This is illustrated in Figure 4.

Initially, the cell culture is mainly composed of subtypes A and B, with a negligible amount of C (Culture 1 in Figure 4A). Subtype C has a better fit with the culture condition and has a higher yield than subtypes A and B. However, under the assumption of no perturbation and perturbation 1 conditions, each with a 50% probability, the projected position of each subtype in the yield–risk space is shown by red symbols. In this case, a combination of subtypes B and C reduces risk without major sacrifice in yield. Because of the covariance of B and C that has a very small positive value (0.068), mitigation of risk over the risk of subtype B is limited. The projected position of each subtype in the yield–risk space will be different if assumed perturbations and their probability of occurrence are different. Under the assumption of no perturbation and perturbation 1 conditions, each with a 50% probability, the projected position of each subtype in the yield–risk space is shown by blue symbols, which are located differently from the red symbols that assume no perturbation for 80% probability. The expected yields of subtypes B and C are now equal; thus, a combination of B and C only reduces risk, yield is not reduced. Yet another assumption of perturbations, in which no perturbation and perturbations 1, 2, 3, and 4 each occur with 20% probability, would result in each subtype being located as shown by the green symbols. In this case, subtypes A and C suffer seriously from perturbations; thus, the population will be dominated by subtype B, which has robust yet low-yield characteristics.
higher yield. Thus, the proportion of subtype C increases over that of subtypes A and B (Culture 2 in Figure 4A). Random mutation gives rise to subtype D. It has higher reproductive potential under this specific culture condition, and thus it quickly proliferates in the population (Culture 3 in Figure 4A). However, when extra perturbations are imposed on the culture, fast-growing but less-robust subtypes (subtype D) may substantially decrease in their proliferation speed or the number of cells. At the same time, low-yield but more-robust subtypes may continue to grow at a similar rate. These population changes result in a composition that better fits a condition with a higher degree of perturbations. In this case, the projected position of the culture on the yield–risk space map may move left to that of Culture 4 in Figure 4A. In contrast, the fast-growing subtype may establish its dominance when the environment reaches a more stable condition that is ideal for the fast but less-robust subtype (Culture 5 in Figure 4A). Both emergence and rebalancing scenarios are included, but for the sake of explanation, only the wild type and its mutational variants are used as subtypes. However, cells with different epigenetic modifications can be considered as subtypes if these modifications affect the yield–risk characteristics of the cell.

Although translation of portfolio theory for biology is shown to be possible, some clear and essential differences have to be made explicit and given a new interpretation that is consistent with biology. First, portfolio selection assumes that there are investors and fund managers making decisions regarding the composition of assets selected for the portfolio. This is clearly not the case in biology. The population composition changes because of the relative growth rate of each cell subtype that is the aggregated effect of individual cell reproduction cycles. Individual cells and organisms will be the subject of selection. Second, in portfolio selection theory, investors decide on the indifference curve to be used on the basis of their appetite for risk taking. In the biological translation, indifference curves are only a reflection of the level of perturbation imposed on organisms and cells (Figure 4B). When organisms and cells are cultured in a highly stationary environment, the use of the risk-neutral curve may best predict their possible evolutionary paths. Risk-aversion curves represent the situation in which perturbations are imposed on organisms and cells.

**Performance suboptimality and robustness trade-offs**

Studies report that suboptimal metabolism performance exists in microorganisms such as *B. subtilis* and *E. coli* (Stelling et al., 2002; Fischer and Sauer, 2005). Fischer and Sauer (2005) argue that several regulatory mutants that have improved biomass production efficiency were ‘almost exclusively regulators of not-yet-activated adaptive responses, suggesting that *B. subtilis* invests valuable resources in anticipation of changing environmental conditions at the expense of optimal growth’. As almost all mutations to enhance biomass production...
Involve genes related to adaptations to environmental changes, it is likely that the projected position of *B. subtilis* is on the efficient frontier and optimized for the risk-aversion indifference curve (orange line in Figure 4B). Type D starts to grow faster than the others and changes the subtype composition of the culture (Culture 3). At this stage, the composition of the culture may be sufficiently optimized for the given culture condition. Suppose the culture condition is changed now to have greater perturbations. Subtype D may not be able to tolerate it and will decrease the rate of proliferation and may even reduce in number, and subtype C may grow faster than the other subtypes (Culture 4). Alternatively, subtype D may continue to grow faster than other subtypes if the environment becomes even more stable. Under the risk-aversion indifference curve, the population arrives at the blue circle on the efficient frontier. The risk-aversion curve represents cases in which higher-level perturbations are imposed on the culture compared with a risk-neutral case. Under the stable condition in which selection pressures other than growth speed are not significant, the risk-neutral indifference curve is likely to be applied. The population follows Trajectory A and maximizes its growth rate at the cost of robustness. Imposing a higher level of perturbation may result in transition of the state through Trajectory B. Cost-free resistance may be a result of taking Trajectory E or F to a new efficient frontier. There may be cases in which the population moves back to suboptimal regions (Trajectories G). Chemotherapy for cancer may shift the point inside the efficient frontier with different end points because of heterogeneous subpopulations. However, tumor cells may again evolve to gain proliferation potential despite the presence of anticancer drugs (Trajectories H). Tumor cells that undergo this transition may be too optimized for this specific therapeutic intervention, which implies possible efficacy when therapeutic regimens are switched. This may explain the collateral sensitivity of drug resistance tumor cells (Skipper et al., 1972).
they can become drug resistant and maintain a competitive growth rate (Andersson, 2006). Although there is controversy with regard to this concept of ‘cost-free evolution,’ if it is correct, this compensatory evolution is thought to adapt to drugs that result in individuals that are more robust against drug insults, without increasing fragility elsewhere or undermining system performance measured by growth rate. In general, pathogens that have acquired drug resistance are known to have less growth fitness than pathogens without drug resistance. This can be seen as a trajectory along the efficient frontier, but toward the lower-left direction in Figure 4B (Trajectory B) and 4C (Trajectory D). Because of the existence of a drug, pathogens that are able to proliferate under drug exposure grow faster than others. Such pathogens may arise because of random mutations that better fit the drug-exposed environment. As a result, the population evolves to be optimal under the strong risk-averse indifference curve. This implies that trade-offs exist between increased drug resistance and a competitive growth rate against nonresistant pathogens. Again, ‘robustness’ is used as a general term to define the organism’s tolerance against perturbations, including environmental fluctuations, the addition of drugs, and mutations. Although acquisition of drug resistance does not affect an organism’s capability to cope with non-drug perturbations, overall robustness was considered to be increased because of added tolerance to the drug. Although the existence of ‘cost-free evolution’ seems to breach the concepts of robustness–performance trade-off, it is consistent because this is a process of moving toward a new efficient frontier (hence, the population is evolving), and robustness–performance trade-off can be observed only on the efficient frontier. The efficient frontier has changed because of the presence of a drug that was not a factor in determining the original efficient frontier (Trajectory E or F in Figure 4C).

Predictions and implications

The application of the concept of portfolio selection to biological systems results in testable predictions. First, it is predicted that the growth rate or biomass production of organisms and cells can be improved through evolution without increasing fragility until the efficient frontier is reached. A trade-off emerges only at the efficient frontier. This can be tested by random sampling of cells in artificial evolution experiments under a stationary culture condition, as shown in Figure 3. By the same token, the growth rate or biomass production of a population of organisms and cells can be improved without increasing their fragility against perturbations. Only when the population is on the efficient frontier do changes in yield and risk affect each other, hence a trade-off emerges. Therefore, robustness (or fragility) is not always conserved; it is conserved only when the system is on the efficient frontier.

Second, once the population or individual cells are on the efficient frontier and a stable culture condition persists, their position in the yield–risk space may move along the efficient frontier to the right for higher yield at the cost of robustness. This is because of the higher growth rate of a subtype over others that better fit the condition. Whether the population is on a trajectory toward the efficient frontier or moving along the efficient frontier can be distinguished by looking at the types of mutations and upregulation of genes that occur during such transitions. If the projected position of the population of cells on the yield–risk space moves toward the efficient frontier from a suboptimal portfolio space (Trajectory C in Figure 4B), mutations and gene up- or downregulations can be observed in broader functional classes of genes. In contrast, if the projected position of the population of cells on the yield–risk space moves along the efficient frontier to the right (Trajectory A in Figure 4B), mutations that generate high-risk high-yield phenotypes and downregulation of genes that accounts for perturbations may be observed. Prediction can be tested by sampling populations of cell and individual cells for sequencing and expression profile measurements to identify distribution of genes that are affected. In addition, different genes may be upregulated in a culture condition in which multiple perturbations are constantly imposed, because this would push the population to a lower-yield projected position. Genes that are accountable for environmental perturbations will be upregulated and genes that obtain a higher yield may be downregulated.

If these conjectures hold and are proven to have wider applicability, there will be several implications for how we handle cell culture experiments. Cells cultured for multiple generations may have the problem of being optimized for a culture-specific condition and higher growth rate rather than for robustness against broader perturbations. Consider the drug-screening process. Drugs are initially screened using cell cultures. When a cancer cell line is used, for example, various drug candidates are applied for various cell lines. Cells in the culture are those that best fit the specific culture condition, which does not necessarily represent an in vivo environment for tumor cells. The most successful drug candidate may then be the one that undermines the growth of cells that are optimized for this specific condition. As in vivo cancer cells may be optimized for surviving under various perturbations, but may not for growth rate, a serious discrepancy exists between cells used for screening and actual cancer cells. Such a discrepancy may be mitigated if a culture condition can be set to impose various perturbations mimicking the cancer cells to which the body may be exposed. Thus, a deeper understanding of the type of perturbations that tumor cells in the body may be exposed to may make it possible to develop a multiple perturbation culture system that may improve the screening process.

By the same token, induced stem cells that are screened for therapeutic purposes may entail a similar problem. Cells with undermined robustness may be selected in favor of efficient reprogramming and upregulation of induction and differentiation markers, rather than cells that maintain robustness against broader perturbations. Currently, multiple generations are required for induction of pluripotent stem cells and elimination of epigenetic traces that are reminiscent of original cells (Masaki et al., 2007). During this process, which often requires multiple passages, new epigenetic modifications that are introduced by specific culture conditions are inevitable (Rubin, 1994; Meissner et al., 2008). It remains to be seen whether characteristics coselected for
high-yield subtypes entail any unwanted characteristics for therapeutic use. With the introduction of the portfolio selection concept, observed breaches of trade-offs and enigma of performance suboptimality can be explained. Further studies and verifications are expected to lead to solid theories for biological systems and their applications to medical research.

Supplementary information
Supplementary information is available at the Molecular Systems Biology website (http://www.nature.com/msb).

Conflict of interest
The author declares that he has no conflict of interest.

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