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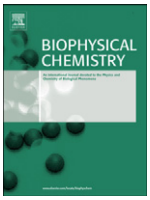

# Interpretation of the Ussing flux ratio from the fluctuation theorem

Chi-Pan Hsieh *

*Department of Physiology, National Taiwan University College of Medicine, No. 1, Jen-Ai Road, 1st Section, Taipei 100, Taiwan*

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ABSTRACT

The fluctuation theorem gives a mathematical expression to quantify the probability of observing events violating the second law of thermodynamics in a small system over a short period of time. The theorem predicts the ratio of forward (entropy-producing) runs to the backward (entropy-consuming) runs for a nanometer-sized molecular machine in a nonequilibrium system. However, few experimental verifications of the theorem have been carried out. In this paper, I show that the Ussing flux ratio, the ratio of outward to inward unidirectional ion fluxes across a membrane channel, can be derived from the fluctuation theorem if we consider the ion channel and the contacting solutions as a small nonequilibrium system. The entropy change due to ion electrodiffusion is expressed from the fundamental equation for the entropy change. Thus, the empirical flux ratio equation can be interpreted from the more general fluctuation theorem, and serves as a verification of the theorem.

## 1. Introduction

The nonequilibrium thermodynamics of small systems have drawn increasing attention in biology as well as physics in recent years, as the behaviors of nanometer-scale biological molecules are studied more and more. A molecular machine has thermodynamic properties distinguishing it from a large machine. When a system's size decreases, thermal fluctuations become significant, and small random deviations from the average behavior may be observable [1]. Molecular machines operate in a Brownian environment, where thermal fluctuations and deterministic forces interplay to accomplish elaborate biophysical and biochemical tasks [2].

The second law of thermodynamics states that the entropy within an isolated system must increase with time during an irreversible process. However, for very small systems over very short times, thermal fluctuations can lead to transient violations of the second law. Nevertheless, the second law is reliable and is not truly violated, as the total entropy must increase if one observes for a long time. The statistical nature of the second law becomes apparent when one considers a small system. When the energy change involved in a small system is comparable to the thermal energy ($k_BT$, the product of Boltzmann's constant and temperature), the molecular machine may run forwards and backwards due to fluctuations [1,3]. In equilibrium, the probability of running forwards equals the probability of running backwards and the net flows of energy and matter are zero. Nonequilibrium systems, which constitute most cases in biology, are more complex, as the net flux follows the thermodynamic driving force. For example, heat flux results from a temperature difference. A volume flow is driven by a pressure gradient. A diffusion flux follows a concentration difference or a chemical potential gradient. In a small nonequilibrium system, there may be a considerable probability of the system running backwards. It would be interesting to know how often such a tiny system runs backwards and violates the second law during a nonequilibrium process, in addition to knowing the direction of the net flux determined by the second law. The question can be answered by the fluctuation theorems developed since the early 1990s.

In 1993, Evans and coworkers formulated the fluctuation theorem that quantified the probability of a second law violation over a short time in dissipative nonequilibrium systems [4,5]. The fluctuation theorem for steady states was developed by Gallavotti and Cohen in 1995 [6]. The fluctuation theorem gives a general mathematical expression for the ratio of the probability of entropy-producing events (forward runs) to the probability of entropy-consuming events (backward runs). The theorem was justified by numerical simulations performed when it was introduced. The first experimental test for the steady-state fluctuation theorem was the study of Rayleigh–Benard convection in 1998 [7]. Evans and coworkers demonstrated their theorem by dragging a colloidal particle with an optical trap and measuring the entropy production/consumption trajectories [8]. Another form of the fluctuation theorem, the Crooks fluctuation theorem, has been verified by stretching single RNA molecules using optical tweezers and calculating the free energies for the folding and unfolding processes [9]. Experimental tests of these theorems have been few because of the difficulties in measuring and differentiating the small entropy-consuming and entropy-producing events. Hence, more experimental evidence is needed to verify the range of applicability of the fluctuation theorem.

In this paper, I wish to apply the fluctuation theorem to an interpretation of the ratio of ion fluxes across cell membranes. As we

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* Tel.: +886 2 23123456x88233; fax: +886 2 23964350.
*E-mail address:* hsiehcp@ntu.edu.tw.

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know, ions flow down their electrochemical gradient across membranes. For more than 50 years, membrane biologists have known that there will be some inward unidirectional flux even when the net flux is outward. The net flux is a result of the difference between the outward unidirectional flux (efflux) and the inward unidirectional flux (influx). The flux ratio can be described by the Ussing flux ratio equation [10], an empirical formula introduced by Ussing in 1949 and supplemented with the flux-ratio exponent proposed by Hodgkin and Keynes in 1955 [11]. The flux ratio equation has been tested using radioactive tracer ions on frog skin [10], squid giant axons [11–13], and *Xenopus* oocyte membranes expressing cloned ion channels [14,15]. Ions flow through ion channels on the membrane by diffusion or electrodiffusion, which are nonequilibrium irreversible processes during which entropy must increase. If we view forward unidirectional flux (down the electrochemical gradient) as entropy-producing and backward unidirectional flux (against the electrochemical gradient) as entropy-consuming, the analogy between the flux ratio equation and the fluctuation theorem becomes explicit. I demonstrate that the Ussing flux ratio can be derived from the fluctuation theorem by using some basic concepts relating diffusional fluxes to entropy production in nonequilibrium thermodynamics. Thus, a more general fluctuation theorem can be applied to interpret a classic empirical formula, and conversely the established flux ratio equation can serve as verification of a modern theorem.

## 2. Theories and models

### 2.1. Ussing flux ratio

Ussing measured unidirectional ion fluxes by tracer ions, and introduced a formula to describe the flux ratio across a membrane [10,16]. For an arbitrary ionic species, $x$, the unidirectional flux from the inside to the outside is $J_{\mathrm{io}}$, and the unidirectional flux from the outside to the inside is $J_{\mathrm{oi}}$. The ratio of the efflux to the influx is

$$\frac{J_{\mathrm{io}}}{J_{\mathrm{oi}}} = \frac{c_{x,\mathrm{i}}}{c_{x,\mathrm{o}}}\exp\left(\frac{zF\Delta\psi}{RT}\right), \tag{1}$$

where $c_{x,\mathrm{i}}$ and $c_{x,\mathrm{o}}$ are the internal and external concentrations of $x$; $z$ is the valence of the ion; $\Delta\psi$ is the membrane potential; and $F$ and $RT$ have their usual thermodynamic meanings. The equation can be expressed equivalently as

$$\frac{J_{\mathrm{io}}}{J_{\mathrm{oi}}} = \exp\left(\frac{zF(\Delta\psi-\psi_x)}{RT}\right). \tag{2}$$

$\psi_x$ is the equilibrium potential of $x$ determined by the Nernst equation.

Hodgkin and Keynes measured the $K^+$ fluxes under a voltage-clamp with the tracer ion $^{42}K^+$ on squid giant axon membranes. The flux ratio in their experiments is described by Eq. (2) raised to the $n$'th power:

$$\frac{J_{\mathrm{io}}}{J_{\mathrm{oi}}} = \left\{\exp\left(\frac{zF(\Delta\psi-\psi_x)}{RT}\right)\right\}^{n'} = \exp\left(\frac{n'zF(\Delta\psi-\psi_x)}{RT}\right). \tag{3}$$

The flux-ratio exponent, $n'$, is about 2.5 to 3.5 in their work and in recent studies on cloned $K^+$ channels expressed in *Xenopus* oocytes [14,15]. A single-file multi-ion long pore was proposed to explain the flux-ratio exponent. Two to three $K^+$ ions must couple with each other in the long pore and move concertedly to transport a tracer ion from one side to the other side [11]. This view is consistent with the crystal structure of the $K^+$ channel pore [17], which was solved in 1998, about 40 years after Hodgkin's insightful work.

### 2.2. The fluctuation theorem

The fluctuation theorem gives a concise general expression for the ratio of the probability, $P(+\sigma)$, of observing a positive entropy production rate, $+\sigma$, to the probability, $P(-\sigma)$, of observing a negative entropy production rate, $-\sigma$, over a short time $t$ [1,4–6]. The entropy production rate is the rate at which the entropy of the system increases with time, $\sigma=\Delta S/t$. The general form of the fluctuation theorem is

$$\frac{P(+\sigma)}{P(-\sigma)} = \exp\left(\frac{\sigma t}{k_{\mathrm{B}}}\right). \tag{4}$$

One can see that there will be a significant probability for observing negative entropy production when $\sigma t$ is small enough to be comparable to $k_{\mathrm{B}}$. The fluctuation theorem provides a verifiable analytical expression for the probability of violating the second law of thermodynamics. When the fluctuation theorem is applied to biological molecular machines, it gives the ratio of forward runs to backward runs [3].

### 2.3. Ion conduction through a single-file multi-ion $K^+$ channel

We use the $K^+$ channel as a model of ion conduction in this paper, though the flux ratio equation can be applied to other types of membrane channels or pores. The understanding of ion conduction through $K^+$ channels has been improved by the structural studies of $K^+$ channels and molecular simulations [18,19]. The selectivity filter of a $K^+$ channel can accommodate 2 to 3 dehydrated $K^+$ ions in a row simultaneously. Ions hop between discrete binding sites in the single-file pore. There are four ion binding sites in the selectivity filter ($S_1$–$S_4$). In addition, there is a weaker binding site for a partially dehydrated $K^+$ ion at the extracellular mouth of the channel ($S_0$). As shown in Fig. 1, the ions jiggle rapidly between the [$S_1$, $S_3$] configuration ($K^+$-water–$K^+$-water) and the [$S_2$, $S_4$] configuration (water-$K^+$–water-$K^+$). The relatively unstable 3-ion configurations also exist during the process of ion conduction. The electrostatic repulsive force between the conducting

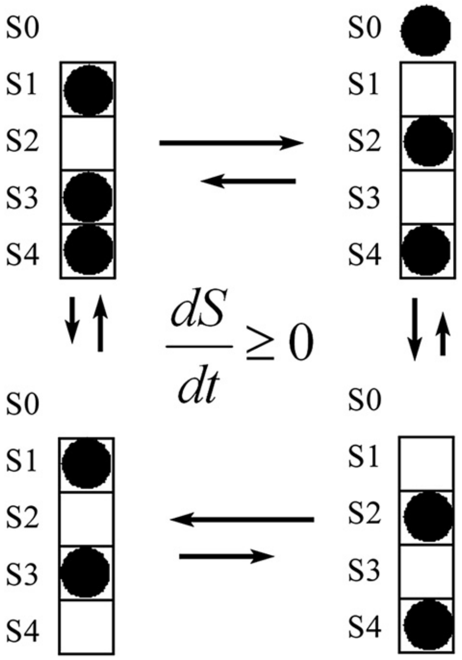

**Fig. 1.** A microscopic model of the transport cycle of ion conduction through a $K^+$channel. The ions jiggle rapidly between the [$S_1$, $S_3$] and [$S_2$, $S_4$] configurations most of the time. An ion transfer occurs when the third ion enters the pore from the intracellular or extracellular solution and knocks off the ion at the opposite end of the pore [18,19]. An outward ion transfer occurs when a clockwise transport cycle is completed. An inward ion transfer takes place when the cycle is completed in the reverse direction. The direction of net flux is determined by the thermodynamic driving force, that is, the electrochemical potential difference across the membrane. Transient backward transport cycle may occur over a short time, although the entropy production must be positive in the long term according to the second law of thermodynamics. The macroscopic flux ratio can be viewed as the ratio of the forward transport duty cycle to the backward transport duty cycle in a single channel.

ions can overcome the attractive force between the ion and the channel pore, and can facilitate the rapid ion transport [17]. Inward ion conduction occurs when a $K^+$ ion binds to the extracellular mouth, dehydrates, and knocks off the inner most $K^+$ ion. Conversely, outward ion conduction occurs when a $K^+$ ion from the intracellular cavity binds to the $S_4$ site and pushes one ion into the extracellular solution [18,19].

It is heuristic to view the ion conduction processes as ion transport cycles (Fig. 1). A net ion is transported from one side to the other side when a cycle is completed. If the single channel conductance is 1 pA ($6\times10^6$ ions/s) at some voltage, $6\times10^6$ net cycles are completed per second. The direction of the net flux depends on the difference in the electrochemical potentials on each side. A forward transport cycle follows the direction the second law predicts, whereas a backward or reverse transport cycle violates the direction required by the second law. At equilibrium potential, the influx equals the efflux; the probability of forward transport equals the probability of backward transport. In nonequilibrium conditions, when the membrane potential is away from equilibrium, there is a net ion flux through the channel, during which the entropy of the system must increase. By the fluctuation theorem, there is some probability for negative entropy production to take place. In the present case, the entropy-consuming event is the backward transport cycle, and the entropy-producing event is the forward transport cycle. As long as the probabilities of positive and negative entropy production can be related to the probabilities of forward and backward transport, the flux ratio equation can be interpreted using the fluctuation theorem.

## 3. Results

To explain how to derive the flux ratio equation from the fluctuation theorem, I first introduce entropy production due to the diffusion of uncharged species in a two-compartment system. Second, I discuss entropy production due to the electrodiffusion of charged species or ions when there is a voltage difference across the membrane of the two-compartment system. Third, I discuss the entropy production in the case of ion conduction through a $K^+$ channel, a single-file multi-ion pore.

### 3.1. Entropy production due to diffusion of uncharged species in an isolated two-compartment system

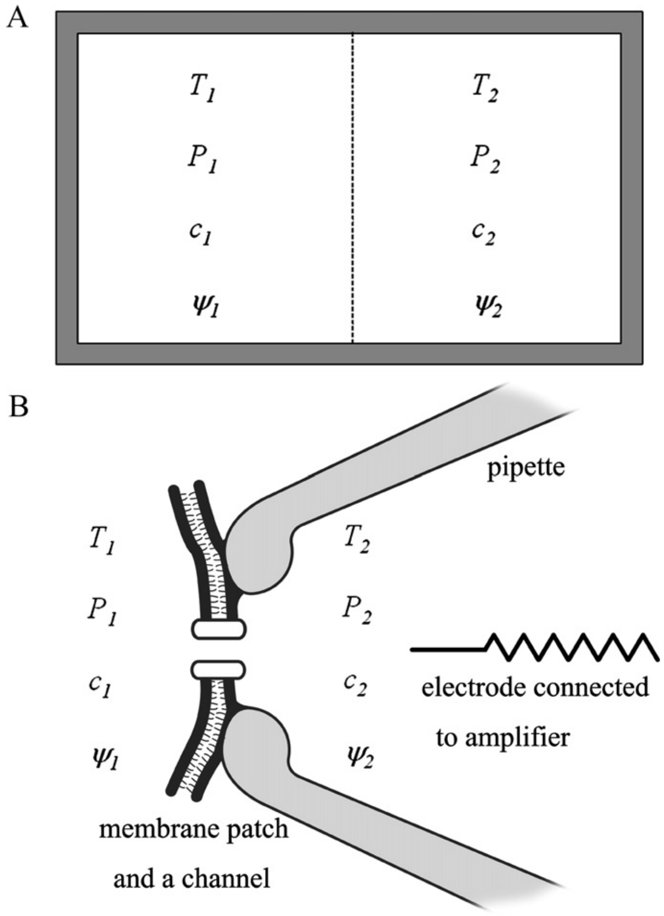

**Fig. 2.** (A) An isolated two-compartment system for considering the entropy change in irreversible processes. The two compartments are separated by a semipermeable membrane which is selectively permeable to one species. The temperature, pressure, concentration and electrical potential are denoted as $T$, $P$, $c$, and $\psi$. (B) Schematic representation of a small system containing an ion channel and the contacting solutions in an inside–out patch-clamp recording. The system is similar to the two-compartment system in (A). In our discussion, we assume $T_1=T_2$ and $P_1=P_2$, the conditions under which the entropy change within the system is due to ion flux by diffusion or electrodiffusion across the channel.

In this section, I discuss entropy production due to the diffusion of uncharged species, or due to the diffusion of ions when the membrane potential is clamped at 0 mV. Diffusion is an irreversible process accompanied by an increase in entropy. One can estimate how much entropy is produced during diffusion from some concepts in nonequilibrium thermodynamics (for details, see [20–22]). Let us consider an isolated two-compartment system separated by a semipermeable membrane, which is selectively permeable to a specific species (Fig. 2A). The concentrations of the species in each compartment are $c_1$ and $c_2$. Since the system is isolated, there is no entropy exchange between the system and the surroundings. All the entropy produced is from the spontaneous irreversible processes within the system. The fundamental equation for the entropy change is

$$dS=\left(\frac{\partial S}{\partial U}\right)_{V,n}dU+\left(\frac{\partial S}{\partial V}\right)_{U,n}dV+\left(\frac{\partial S}{\partial n}\right)_{U,V}dn, \tag{5}$$

or the differential form,

$$dS=\frac{dU}{T}+\frac{PdV}{T}-\frac{\mu dn}{T}. \tag{6}$$

Here $U$ is the internal energy; $P$ is the pressure; $V$ is the volume; $n$ is the number of molecules; and $\mu$ is the chemical potential of the diffusing matter. Eq. (6) gives the thermodynamic driving forces for the fluxes: $1/T$ is the tendency for heat, $P/T$ is the force for volume flow, and $\mu/T$ is the driving force for diffusion. For the discontinuous two-compartment system, the total entropy produced is the sum of the entropy change of each compartment:

$$dS=\frac{dU_1}{T_1}+\frac{dU_2}{T_2}+\frac{P_1dV_1}{T_1}+\frac{P_2dV_2}{T_2}-\frac{\mu_1dn_1}{T_1}-\frac{\mu_2dn_2}{T_2}. \tag{7}$$

Since $dU_1=-dU_2$, $dV_1=-dV_2$, $dn_1=-dn_2$,

$$dS=\left(\frac{1}{T_1}-\frac{1}{T_2}\right)dU_1+\left(\frac{P_1}{T_1}-\frac{P_2}{T_2}\right)dV_1-\left(\frac{\mu_1}{T_1}-\frac{\mu_2}{T_2}\right)dn_1. \tag{8}$$

Eq. (8) must always produce a positive result because by the second law $dS$ must be positive in irreversible processes within an isolated system. Let us consider a specific condition when $T_1=T_2$ and $P_1=P_2$, a reasonable condition for a patch-clamp recording. Since I am discussing the entropy change due to diffusion of an uncharged species, the contribution of the electrical potential is neglected ($\psi_1=\psi_2=0$ mV). Then, the change in entropy is

$$dS=-\left(\frac{\mu_1-\mu_2}{T}\right)dn_1\geq0. \tag{9}$$

Here entropy is produced by diffusion only. The net direction of the diffusion flux is determined by the difference in the chemical potentials of the two compartments. When $\mu_1>\mu_2$, the matter is transported from compartment 1 to compartment 2. The chemical potential of a molecule in an ideal or dilute solution is

$$\mu = \mu^{\mathrm{o}} + k_{\mathrm{B}} T \ln c. \tag{10}$$

It should be noted that for a real solution, the chemical potential would deviate from ideality. According to the Debye–Hückel theory of ionic solutions, the chemical potential of the ion is lowered by the electrostatic interaction between ions. In practice, the activity or “active concentration”, $a$, is commonly introduced for a real solution. The chemical potential for a real solution is $\mu=\mu^{\mathrm{o}}+k_{\mathrm{B}}T \ln a=\mu^{\mathrm{o}}+k_{\mathrm{B}}T \ln \gamma c$, where $\gamma$ is the activity coefficient. Though the concentration, $c$, is used in Eq. (10) and in the following discussion for consistency, one should know that the activity of ions should be considered in a real solution.

By substituting Eq. (9) into Eq. (2), one gets the change in entropy

$$\mathrm{d}S = -k_{\mathrm{B}} \ln \frac{c_1}{c_2} \mathrm{d}n_1. \tag{11}$$

Now one can answer how much entropy ($\Delta S$) is produced when an uncharged molecule is transported forward by diffusion across the membrane from compartment 1 to compartment 2 in a small system over a short time $t$:

$$\Delta S = -k_{\mathrm{B}} \ln \frac{c_1}{c_2}(-1) = k_{\mathrm{B}} \ln \frac{c_1}{c_2}. \tag{12}$$

The fluctuation theorem predicts the ratio of the probability of observing a positive entropy production rate, $+\sigma$, to the probability of observing a negative entropy production rate, $-\sigma$, over a short time $t$. By substituting Eq. (12) into Eq. (4), we have

$$\frac{P(+\sigma)}{P(-\sigma)} = \exp\left(\frac{\sigma t}{k_{\mathrm{B}}}\right) = \exp\left(\frac{\Delta S}{k_{\mathrm{B}}}\right) = \exp\left(\frac{k_{\mathrm{B}} \ln \frac{c_1}{c_2}}{k_{\mathrm{B}}}\right) = \frac{c_1}{c_2}. \tag{13}$$

In the case of ion conduction through a single channel under a voltage-clamp at 0 mV, the above ratio can be interpreted as the ratio of forward transport cycles to backward transport cycles. The ratio is the Ussing flux ratio (Eq. (1)) when $\Delta\psi=0$. The flux ratio at 0 mV is simply the ratio of the ionic concentrations.

### *3.2. Entropy production due to electrodiffusion of ions in an isolated two-compartment system*

Let us now consider the isolated two-compartment system when there is a difference in electrical potentials across the membrane. The electrical potentials of compartment 1 and compartment 2 are $\psi_1$ and $\psi_2$. The two compartments are separated by a membrane selectively permeable to only one species of ion. The ion flux is determined by the concentration gradient and the electrical potential difference. The change in the entropy can be written as [20,21]:

$$\mathrm{d}S = \frac{\mathrm{d}U}{T} + \frac{P\mathrm{d}V}{T} - \frac{(\mu + ze\psi)\mathrm{d}n}{T} = \frac{\mathrm{d}U}{T} + \frac{P\mathrm{d}V}{T} - \frac{\tilde{\mu}\mathrm{d}n}{T}, \tag{14}$$

where $\tilde{\mu}$ is the electrochemical potential,

$$\tilde{\mu} = \mu + ze\psi. \tag{15}$$

$e$ is the elementary charge. It is useful and practical to introduce the electrochemical potential in the electrochemical system. Guggenheim suggested that the electrochemical potential should be treated as a primary thermodynamic quantity [20,23], because the chemical and the electrical potentials cannot be separated experimentally. Again, let us assume $T_1=T_2$ and $P_1=P_2$. The total increase in the entropy of the two-compartment electrochemical system is

$$\mathrm{d}S = -\frac{\tilde{\mu}_1 \mathrm{d}n_1}{T} - \frac{\tilde{\mu}_2 \mathrm{d}n_2}{T} = -\left(\frac{\tilde{\mu}_1 - \tilde{\mu}_2}{T}\right)\mathrm{d}n_1, \tag{16}$$

where $\mathrm{d}n_1=-\mathrm{d}n_2$. The direction of net ion flux by electrodiffusion is determined by the electrochemical potential difference of the two compartments. Ion flows from the compartment with higher electrochemical potential to the compartment with lower electrochemical potential. The electrochemical potential of an ion in the dilute solution is (Eqs. (10), (15))

$$\tilde{\mu} = \mu^{\mathrm{o}} + k_{\mathrm{B}} T \ln c + ze\psi. \tag{17}$$

Substituting Eq. (17) into Eq. (16) gives,

$$\mathrm{d}S = -\left(k_{\mathrm{B}} \ln \frac{c_1}{c_2} + \frac{ze}{T}(\psi_1 - \psi_2)\right)\mathrm{d}n_1. \tag{18}$$

Now I consider the small system of a single channel and its adjacent solutions. The change in the entropy when an ion is transported across the membrane from compartment 1 to compartment 2 is

$$\Delta S = -\left(k_{\mathrm{B}} \ln \frac{c_1}{c_2} + \frac{ze}{T}(\psi_1 - \psi_2)\right)(-1) = k_{\mathrm{B}} \ln \frac{c_1}{c_2} + \frac{ze}{T}(\psi_1 - \psi_2). \tag{19}$$

By the fluctuation theorem, the ratio of the forward transport cycles to the backward transport cycles is,

$$\frac{P(+\sigma)}{P(-\sigma)} = \exp\left(\frac{\sigma t}{k_{\mathrm{B}}}\right) = \exp\left(\frac{\Delta S}{k_{\mathrm{B}}}\right) = \frac{c_1}{c_2} \exp\left(\frac{ze(\psi_1 - \psi_2)}{k_{\mathrm{B}} T}\right) = \frac{c_1}{c_2} \exp\left(\frac{zF\Delta\psi}{RT}\right), \tag{20}$$

where $\Delta\psi=\psi_1-\psi_2$. The ratio is exactly the same as Ussing flux ratio (Eq. (1)). I have demonstrated how to derive the flux ratio equation from the fluctuation theorem.

### *3.3. Flux ratio in the K$^+$ channel, a single-file multi-ion pore*

My goal is to express the entropy production in a small system like a single-channel recording configuration under voltage-clamp (Fig. 2B). The system consists of a single K$^+$ channel on the membrane, and extracellular and intracellular ionic solutions contacting the channel. The electrical potentials of both sides are fixed. The local K$^+$ concentrations, which are in equilibrium with the concentrations of the reservoir solutions, can be considered to be fixed, because the number of ions transported between the solutions is negligible compared with the number of K$^+$ ions in the bulk solutions. Hence, the membrane potential and the K$^+$ concentrations are control parameters in the system. In this nonequilibrium steady-state system, the ion flux is a fluctuating variable. The fluctuating trajectories of the ion flux can be revealed with nanosecond resolution in Brownian dynamics simulations [18], though only a steady net current can be recorded in a single-channel patch clamp recording due to the limited time resolution of the electrical signals.

The patch-clamp recording configuration is similar to the two-compartment system described in the previous section, with an electrical potential difference across the membrane. Experiments using tracer ions have shown that the flux ratio of K$^+$ channels deviates from the Ussing flux ratio (Eq. (1)) [11,13–15]. The flux ratio in K$^+$ channels is Ussing's equation raised to $n$'th power (Eq. (3)). The flux-ratio exponent, $n'$, is about 2.5 to 3.5 in experiments on K$^+$ channels. The single-file multi-ion feature of the K$^+$ channel accounts for the origin of the flux-ratio exponent [11]. The narrow selectivity filter of a K$^+$ channel can accommodate 2 to 3 K$^+$ ions. As shown in Fig. 1, ions would pause briefly in a certain configuration, and when they gain enough energy from the Brownian environment, 2 or 3 ions hop over the energy barrier to transit to another configuration [16]. A feature of ion channels is their rapid transport rate, approaching the diffusion limit. The pause in each configuration is very short; the translocation between configurations takes place in nanosecond [18]. The ion transport cycle can run forwards and backwards with discrete steps. An ion transfer event occurs after a transport cycle is complete.

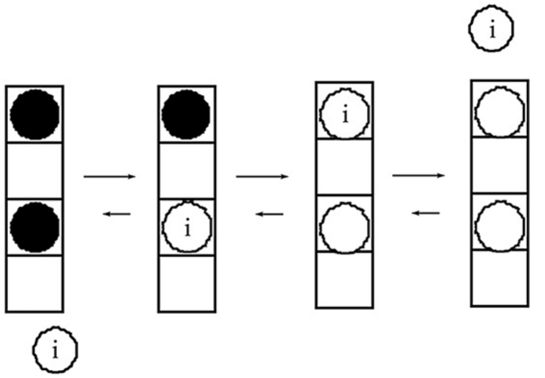

**Fig. 3.** The process for transferring a labeled tracer ion across the single-file multi-ion pore. We suppose that the intracellular ions are all labeled ions (white). Note that each step in this figure is a result of a complete transport cycle in Fig. 1. It takes 3 net outward transport cycles to transport a specific labeled ion (i) from the inside to the outside in a 2-ion pore. Thus in the tracer ion experiment, a flux-ratio exponent of 3 indicates that the single-file pore may accommodate 2 ions at a time.

Let us imagine that the $K^+$ ions in the intracellular solution are all labeled ions, and the single-file pore holds $m$ ions at a time; then it takes $m+1$ more cycles in the outward direction than in the inward direction to transfer a labeled ion from inside to outside (Fig. 3). The flux-ratio exponent, $n'$, implies that $n'$ more forward cycles than backward cycles are needed to transport a tracer ion across the channel. A value of ~3 for $n'$ from experiments indicates that there are about 2 $K^+$ ions in the $K^+$ channel pore at a time, consistent with images from the crystal structure of the $K^+$ channel [17,19]. If the entropy change for transporting an ion is $\Delta S$ (Eqs. (16), (19)), the entropy change for a tracer transfer event will be $n'\Delta S$. By the fluctuation theorem, we can estimate the probability of the backward tracer transfer event violating the second law.

$$\frac{P(+\sigma)}{P(-\sigma)} = \exp\left(\frac{n'\Delta S}{k_{\mathrm{B}}}\right) = \exp\left(\frac{n'zF(\Delta\psi-\psi_x)}{RT}\right). \tag{21}$$

The larger the flux-ratio exponent, the less likely a tracer ion can be transported across the channel against the electrochemical driving force. For example, when $\Delta\psi-\psi_x=25$ mV at room temperature, the likelihood of a reverse tracer transfer event is 37% if $n'=1$, and is only 5% if $n'=3$.

## 4. Discussion

I demonstrate that the ratio of ion fluxes can be interpreted from the fluctuation theorem. The flux ratio is determined by intensive external parameters: ion concentrations, membrane potentials, and temperature, which are measurable in the solutions on both sides of the membrane. Intriguingly, the ratio is less dependent on internal parameters, such as the profile of the energy barrier, kinetic rates, viscosity, and the resistance of the channels, even though these parameters affect the magnitude of the fluxes. Previous permeation models have found that the kinetic rate constants are cancelled out in the process of deriving the flux ratio equation [24,25]. The flux ratio equation is also independent of the type of channel; it has been applied to channels for different ions and to gramicidin pores [16], although I use only the $K^+$ channel as the example in this paper. As is well known, one needs only external parameters to describe an equilibrium system. However, nonequilibrium systems usually require some internal parameters for describing fluxes, such as the heat transfer coefficient for heat flux, the resistance for the electrical currents in a circuit, or the conductance for the ion flux of a channel. The absence of internal parameters in the flux ratio equation is reasonable when we interpret it from the fluctuation theorem. The ion–ion interactions within the channel and the ion–channel interactions would not affect the interpretation and the results presented in this paper.

As is shown above, the flux ratio can be derived from the more general fluctuation theorem without specifying internal parameters. Although the entropy production rate, $\sigma$, may be affected by kinetic parameters and may be different in various ion channels, the ratio of $P(+\sigma)$ to $P(-\sigma)$ is determined by the change in entropy $\Delta S=\sigma t$. As Eq. (16) indicates, the entropy change for transporting a single ion through the channel depends only on the difference of the electrochemical potentials of the solutions. Hence, the flux ratio, as well as the ratio of $P(+\sigma)$ to $P(-\sigma)$, is determined by the electrochemical activities of the solutions, and is less dependent on the internal mechanical properties of the channels. It seems unsatisfactory to regard the exponential function in the flux ratio equation as a result of the Boltzmann distribution [18], which describes the distribution of occupancies of equilibrium states, because ion fluxes are nonequilibrium quantities.

The flux-ratio exponent, $n'$, was attributed to the single-file multi-ion feature of a long pore by Hodgkin and Keynes with foresight in 1955 [11], more than 40 years before the first X-ray crystal structures of $K^+$ channel pore were solved in 1998 [17]. As aforementioned, a tracer transfer from one side to the other requires $n'$ more transport cycles in one direction than in the other direction. It appears to be improper to regard $n'e$ as the valence of the multiple ions in the pore [26]. When there is no electrical potential difference across the membrane ($\Delta\psi=0$), or when we consider the diffusion of uncharged species, the flux ratio is simply $(c_1/c_2)^{n'}$, in which case the valence of ions is not necessary for the existence of the flux-ratio component.

Scientists have been considering for more than a century that thermal fluctuations can lead to transient violations of the second law of thermodynamics when the system is sufficiently small. In 1871, Maxwell introduced a hypothetical finite being, later called Maxwell's demon, to explain the limitation of the second law in a small system [27]. By the brilliant analysis of Smoluchowski's trapdoor [28,29] and Feynman's Brownian ratchet [30], a small gadget cannot use the fluctuations from the surroundings to undergo perpetual motion in an equilibrium system because the gadget itself will experience thermal fluctuations with the same chance as it can get thermal energy from the surroundings. Hence the machine just runs forwards and backwards and gains no net directed motion. However, it is possible for a specially designed molecular machine, for example, the semiconductor p–n diode, or Feynman's Brownian ratchet, to harness the fluctuation from the environment more easily in one direction than in opposite direction in a nonequilibrium system.

Thermal fluctuations set a thermodynamic limit on the reflection near the reversal potential in the p–n diode. Backwards runs or steps are inevitable in a regime not far from equilibrium. The current–voltage (*I-V*) curve never bends sharply but rectifies smoothly near the reversal point [31]. In this paper, we interpret the flux ratio in ion channels as the ratio of discrete forward transport cycles to reverse transport cycles from the fluctuation theorem. Ion channels can be regarded as Brownian ratchets. Some ion channels have rectified *I-V* curves near the reversal potential. It would be interesting to test experimentally whether the same flux ratio equation holds over the rectified nonlinear regime near the equilibrium potential, as the fluctuation theorem is said to be valid in the nonlinear regime. There may be some common principles underlying the mechanisms of rectification for the ion channel, the p–n diode, and Feynman's ratchet.

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