

Time Irreversibility, Entropy Production, and Effective Temperature Are Independently Regulated in the Actin Cortex of Living Cells

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Living cells exhibit nonequilibrium dynamics emergent from the intricate interplay between molecular motor activity and the viscoelastic cytoskeletal matrix. The deviation from thermal equilibrium can be quantified through frequency-dependent effective temperature or time-reversal symmetry breaking quantified, e.g., through the Kullback-Leibler divergence. Here, we investigate the fluctuations of an AFM tip embedded within the active cortex of mitotic human cells with and without perturbations that reduce cortex activity through inhibition of material turnover or motor proteins. While inhibition of motor activity significantly reduces both effective temperature and time irreversibility, inhibited material turnover leaves the effective temperature largely unchanged but lowers the time irreversibility and entropy production rate associated with the fluctuation-dissipation theorem violation of tip dynamics. Our experimental findings in combination with a minimal model highlight that time irreversibility, effective temperature, and entropy production rate can follow opposite trends in active living systems, challenging, in particular, the validity of effective temperature as a proxy for the distance from thermal equilibrium, particularly in the presence of mechanical changes. Furthermore, we propose that biological activity regulates the occurrence of time-asymmetric deflection spikes in the dynamics of observables, providing a previously unrecognized link between entropy production and time irreversibility.

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I. INTRODUCTION

Mechanics and statistical physics describe the laws governing the motion of objects at mesoscopic and macroscopic scales in thermodynamic equilibrium. Living cells are paradigmatic nonequilibrium systems due to their continuous consumption and dissipation of energy. To characterize the dynamics of living cells, methods that quantify deviations from equilibrium are essential. In equilibrium, the fluctuation-dissipation theorem (FDT) relates the response function of a physical variable to the strength of its fluctuations. In active systems, deviations from this relation provide a direct verification of nonequilibrium activity.

A prevalent approach to quantify this violation is the definition of an effective temperature T_{eff} whose excess over the ambient temperature reflects the degree to which fluctuations surpass thermal levels. The effective temperature is a

function of frequency that has been intensively studied, both theoretically and experimentally—for instance, in synthetic active systems that are glassy or granular [1,2]. In the past decades, the idea has also been extended to biological systems, e.g., the active cytoskeleton [3] and hair bundles of sensory hair cells in the inner ear [4], where the violation of the FDT in the form of higher effective temperature is observed at smaller frequencies ($\lesssim 10$ Hz). At higher frequencies, these systems are governed by the temperature of the bath. This is due to the fact that the underlying molecular motors which impart active forces operate at smaller frequencies [5]. However, violations of the FDT can also occur at higher frequencies, as demonstrated for discrete Markov systems [6].

In addition to effective temperature, the asymmetry of underlying active dynamics under time reversal, e.g., quantified by the Kullback-Leibler divergence (KLD), serves as a second important indicator of activity [7–13].

Previous biophysics research has used effective temperature T_{eff} or time irreversibility as a measure of deviation from equilibrium, implying a monotonic increase of these measures with entropy production rate; see, e.g., Refs. [5,7,14–20]. However, direct experimental validation of this assumption remains elusive. To fill this gap, we provide here a comparative study of both effective

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temperature and time irreversibility in relation to entropy production rate applied in an exemplary manner to the dynamics of an AFM tip embedded in the active actin cortex—a thin biopolymer network underneath the plasma membrane of animal cells.

II. ACTIVE FLUCTUATIONS OF CORTEX-EMBEDDED AFM TIPS

To experimentally assess the active mechanics of the cell cortex, we choose to work with HeLa cells arrested in mitosis. Such cells are particularly suitable for our study, as they naturally adopt an almost spherical shape with a strongly activated molecular motor activity in the actin cortex [21,22]. Furthermore, we arrested the cells in mitosis to prevent temporal changes of material properties due to progression of cells through the cell cycle; see Sec. VI. During our measurements, we embedded the tip of a pyramidal AFM cantilever in the cortex. A schematic sketch representing the experimental assay is depicted in Fig. 1(a). A snapshot (bottom view) of a mitotic cell being probed by the AFM cantilever is shown in Fig. 1(b). Since the embedded AFM tip gets mechanically coupled to the actin cortex, its deflection serves as an effective readout of local active fluctuations within the cortical layer.

In Fig. 1(c), we present a representative time evolution of the cantilever hinge height h alongside the vertical tip deflection $\delta_c = F/\kappa$, where F denotes the measured AFM force and κ is the cantilever's spring constant. Once the set-point force is reached ($t \approx 20$ s), the hinge height remains constant, while the tip deflection exhibits an exponential-like relaxation—a characteristic response of a viscoelastic material [23]. Subsequently, the tip position fluctuates around $\delta_c = 0$, driven by a combination of thermal and athermal forces acting on the system.

Strikingly, the tip position exhibits pronounced active bursts directed outward from the cell (upward tip motion), with amplitudes of approximately $A_u \approx 10$ nm and a subsequent relaxation which is captured by an exponential decay with timescale of $\tau \approx 1.5$ s; see Figs. S1(a)–S1(d) [24]. A magnified view of an upward active burst is shown in the left inset in Fig. 1(c). We note that these bursts are inherently asymmetric in time; see also Figs. S1(c)–S1(g) [24]. Moreover, the tip also undergoes substantial deflection events directed toward the cell interior, as shown in the inset in Fig. 1(c). However, these events have smaller amplitudes $A_d \approx 5$ nm and exhibit a less asymmetric shape; see Figs. S1(f) and S1(i)–S1(o), respectively [24]. In addition to this, the active nature of the tip fluctuations is also evident from the probability distribution function of tip

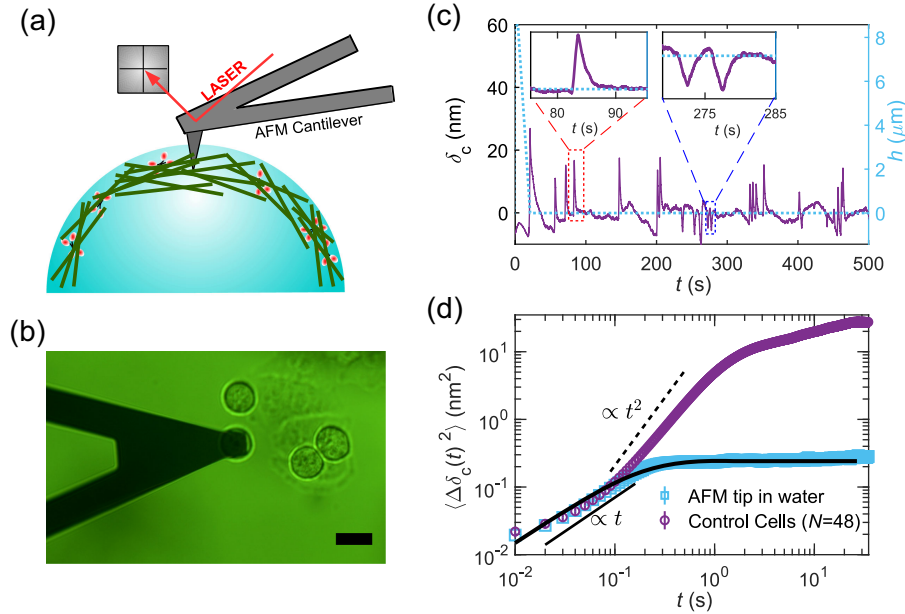


FIG. 1. Probing active cortex fluctuations with AFM. (a) Schematic illustration of the experiment (green, actin polymers; red, motor proteins). (b) Microscopy image showing cells and an AFM cantilever during the measurement (bottom view). The pyramidal AFM cantilever tip is embedded in the cortex of a HeLa cell in mitotic arrest from the top. Scale bar: 20 μ m. (c) Left: a typical experimental time progression of the tip position $\delta_c(t)$ embedded within the cortex. Right: corresponding piezo-controlled hinge height $h(t)$ as a function of time. For simplicity, final hinge height and the mean of $\delta_c(t)$ were set to zero. Left inset: enlarged view of an active force burst directed toward the cantilever displaying a sudden increase of the tip position followed by an exponential-like relaxation. Right inset: expanded view around an active force burst directed toward the interior of the cell with an approximately time-symmetric shape. (d) Time dependence of the measured mean-squared displacement of a tip inserted within the cortex of mitotic cells (purple circles) and for the tip suspended in aqueous medium (blue square). The black solid curve represents a fit to Eq. (1) with $\kappa = 0.0355$ N/m and $\gamma = 0.0056$ Ns/m.

displacements $P(\Delta\delta_c)$ at various time lags which exhibit strongly non-Gaussian tails [2,28], as shown in Fig. S2 [24].

To quantify the active fluctuations described above, we computed the mean-squared displacement (MSD) of the tip position, $\langle\Delta\delta_c(t)^2\rangle = \langle[\delta_c(t+t_0) - \delta_c(t_0)]^2\rangle$. For AFM cantilevers freely fluctuating in cell culture medium, tip fluctuations can be described as that of an overdamped harmonic oscillator in a viscous medium:

$$\langle\Delta\delta_c^2(t)\rangle = \frac{2k_B T}{\kappa} \left[1 - \exp\left(-\frac{\kappa t}{\gamma}\right) \right], \quad (1)$$

where γ represents the viscous friction coefficient; see Fig. 1(d), blue curve. Correspondingly, the MSD converges to a plateau $2k_B T/\kappa$ with κ approximately the nominal spring constant of the cantilever and T the ambient bath temperature. Notably, fluctuations of AFM cantilevers embedded in the active cell cortex were strongly enhanced as compared to cantilevers freely immersed in medium; see the purple curve in Figs. 1(d) and S3 [24]. Furthermore, the MSD of cortex-immersed tips displays a transition from a diffusive behavior at short times $t \lesssim 0.1$ s to superdiffusive dynamics for $0.1\text{ s} \lesssim t \lesssim 1$ s, a typical signature of the active nature of the cortex [29]. Because of the caged motion of the AFM tip, the MSD converges to a plateau $\langle\Delta\delta_c(t \rightarrow \infty)^2\rangle = 2k_B T_{\text{eff}}/\kappa$, where T_{eff} represents the effective temperature in the long-timescale limit [30,31]. Using the measured plateau value, we can estimate the effective temperature T_{eff} to be about 2 orders of magnitude higher than the ambient bath temperature $T \approx 310$ K of the cells.

III. EFFECTIVE TEMPERATURE AND ENTROPY PRODUCTION

The actin cortex is subject to constant ATP-driven polymer turnover and active forces exerted by associated motor proteins [32]. To dissect the effect of these processes on active fluctuations, we applied low doses of latrunculin-A (25 nM) to slow down actin polymerization and (-)blebbistatin (10 μM) to reduce active myosin forces [33,34]. In Fig. 2(a), we plotted the measured MSD of tip fluctuations subject to the above-mentioned drug treatments along with measurement results of untreated cells (control) and of the bare tip in aqueous medium. In comparison to control, the MSD for both treatments exhibits quantitatively distinct long-time behavior with a lowered plateau in the long-time limit reflecting reduced cortical activity.

To better quantify the out-of-equilibrium nature of tip fluctuations in different situations, we investigated the dynamics using the FDT [35,36]:

$$\frac{C(f)}{2k_B T} = \frac{\chi''(f)}{2\pi f}, \quad (2)$$

where f is the frequency of fluctuations. As described in Sec. VI, the FDT relates the power spectral density $C(f)$ of tip fluctuations $\delta_c(t)$ to the imaginary part of the response function $\chi''(f)$. The latter was determined via active sinusoidal cantilever oscillations at frequencies $f = 0.02\text{--}10$ Hz; see Sec. VI. The obtained results for $\pi f C(f)/(k_B T)$ (open symbols) and $\chi''(f)$ (filled symbols) are plotted in Fig. 2(b). The violation of the FDT for a specific frequency is marked by the ratio between the two quantities, i.e., $T_{\text{eff}}(f)/T$. Our experiments clearly show a violation of the FDT for frequencies $f \lesssim 1$ Hz as indicated by $T_{\text{eff}}(f)/T > 1$ in this regime; see Fig. 2(c). This observation confirms the active nature of the measured tip fluctuations within the cortex, suggesting that cell cortex activity predominantly arises from relatively slow biological processes, such as myosin activity, actin polymerization, and blebbing kinetics [5,14,37–40]. For $f \gtrsim 1$ Hz, the fluctuations are dominated by passive thermal noise. Consistent with the observed MSD, the degree of violation is more pronounced for control cells (purple circles) and cells with turnover inhibition (green diamonds), as compared to cells with inhibited motor activity (yellow triangles). Interestingly, the measurement curves of effective temperature versus frequency are well captured by an exponential decay of effective temperature with characteristic timescale of 2/3 s; see Fig. S13 [24].

Earlier research demonstrated that the effective temperature in a viscoelastic fluid is linked to the energy dissipation spectrum $I(f)$ of the observed dynamics by the Harada-Sasa equality [41–43]

$$I(f) = \frac{2k_B [T_{\text{eff}}(f) - T]}{1 + [G'(f)/G''(f)]^2}. \quad (3)$$

The corresponding entropy production rate is $\sigma = \int df I(f)/T$. Here, $G'(f)$ and $G''(f)$ represent the real and imaginary components, respectively, of the frequency-dependent complex shear modulus $G^*(f)$ of the actin cortex, where the real part characterizes the solidlike response and the imaginary part captures the fluidlike response. Accordingly, $I(f)$ vanishes for $T_{\text{eff}}(f) = T$, which corresponds to the thermal equilibrium scenario. Additionally, $I(f)$ depends on the mechanical properties of the surrounding environment characterized by the inverse loss tangent $G'(f)/G''(f)$, which is a measure of solidity. This functional dependence acknowledges that only the fluidlike response of a material gives rise to energy dissipation, while in a purely solidlike material fluctuations merely reflect time-dynamic exchange between kinetic and elastic energy.

Performing active rheological measurements (see Fig. S4 [24] and Sec. VI), we determined the complex elastic modulus $G^*(f)$; see Fig. S5 [24] and Sec. VI. Thus, we can calculate $G'(f)/G''(f)$; see the inset in Fig. 2(d). Notably, we find a marked increase of $G'(f)/G''(f)$ for

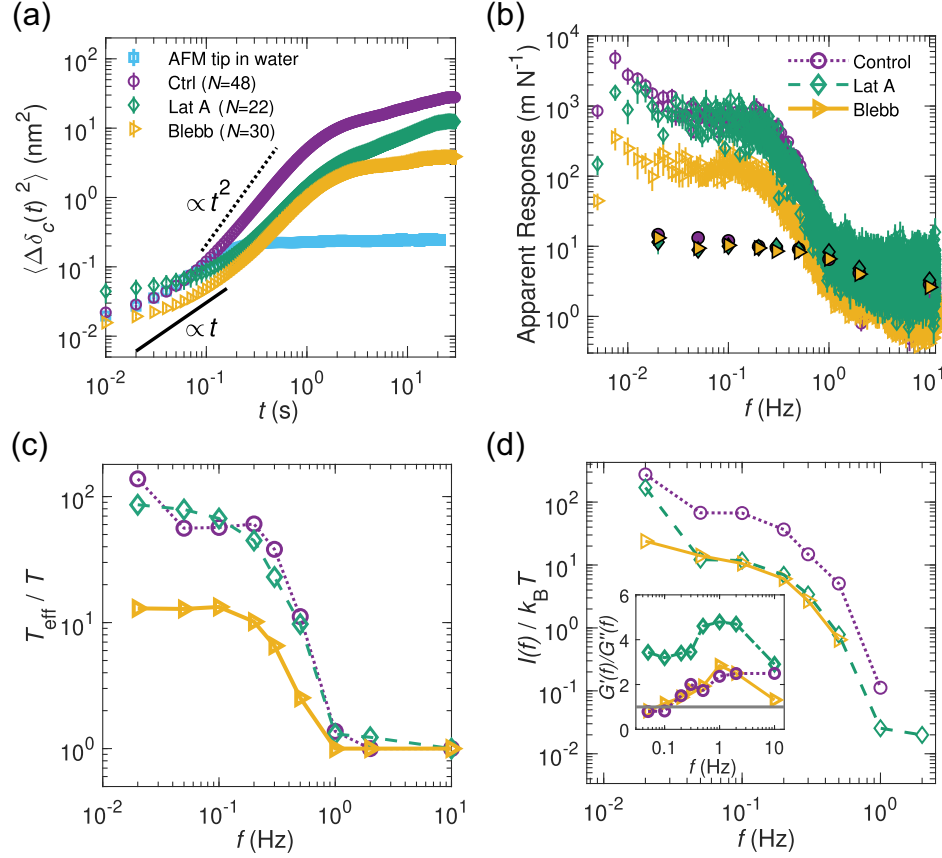


FIG. 2. Enhanced effective temperature highlights the active nature of AFM tip fluctuations in the actin cortex. (a) Experimentally measured MSD $\langle \Delta \delta_c(t)^2 \rangle$ for the tip suspended in aqueous medium at $T = 310$ K and inserted within the cortex of the mitotic cells under control (purple circles) and pharmacologically treated conditions: 10 μM blebbistatin (yellow triangles) and 25n M latrunculin-A (green diamonds). (b) Deviation from the fluctuation-dissipation theorem [Eq. (2)]. The open symbols represent $\pi f C(f)/k_B T$, while the filled symbols correspond to the imaginary part of the response function, $\chi''(f)$. Symbols and color codes are as in (a). (c) Effective temperature T_{eff} normalized by the ambient temperature as a function of frequency obtained from the violation of the FDT from (b) for control (purple circles), treated with latrunculin-A (green diamonds) or blebbistatin (yellow triangles). (d) Energy dissipation spectrum $I(f)$ obtained from the fluctuations of AFM cantilever tips embedded in the mitotic actin cortex. Symbols and color codes are as in (a). Inset: experimentally measured ratio of storage modulus to loss modulus, $G'(f)/G''(f)$, as a function of frequency, characterizing the solidlike nature of the actin cortex. The gray curve represents the reference line where $G'(f)/G''(f) = 1$, indicating the transition between predominantly elastic and viscous behavior.

latrunculin-treated cells where actin material turnover is inhibited. Using results for $G'(f)/G''(f)$, we determine $I(f)$ for both control and treated cells; see Fig. 2(d). Because of the more pronounced solidlike nature of latrunculin-treated cells, dissipation is reduced as compared to blebbistatin-treated cells. Thus, while the latrunculin-treated cells exhibit a larger effective temperature T_{eff} compared to the blebbistatin-treated cells, their dissipation spectra $I(f)$ are similar. By integrating the normalized dissipation spectrum over the entire frequency range, we obtain the following entropy production rates associated with the violation of the FDT of the observed tip dynamics: $\sigma = 19.58 k_B/\text{s}$ for control cells, $5.51 k_B/\text{s}$ for latrunculin-treated cells, and $2.94 k_B/\text{s}$ for blebbistatin-treated cells. We conclude that, upon turnover inhibition (latrunculin-A treatment), the entropy production rate

decreases significantly, while the effective temperature remains largely unchanged.

IV. TIME IRREVERSIBILITY FOLLOWS ENTROPY PRODUCTION TRENDS

Recently, a novel measure $\hat{\sigma}$ for the nonequilibrium nature of a system was introduced based on the quantification of time irreversibility [8,44]. This measure captures the divergence between the probabilities of experimentally observed forward and reverse trajectories in terms of the KLD of whitened residual signals. Following the approach in Ref. [7], we compute the residual time series $\zeta^F(t)$ and $\zeta^R(t)$ from measured time series; see Figs. 3(a)–3(d) and Sec. VI. After whitening, the autocorrelation function of the residuals vanishes at all times, indicating that the

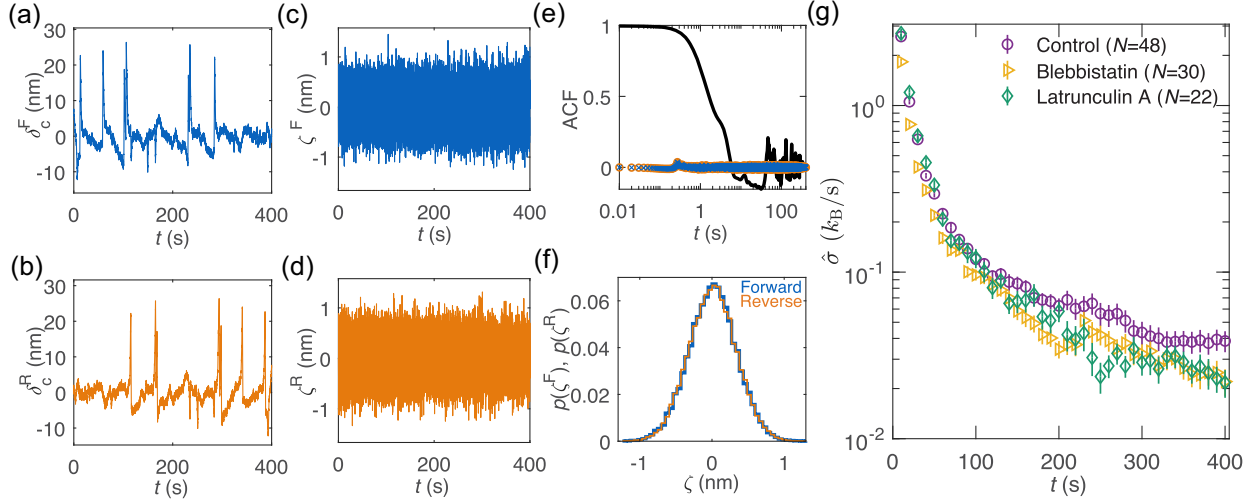


FIG. 3. Inhibition of actin turnover and motor activity reduces time irreversibility. (a), (b) Representative steady-state fluctuations of the AFM tip immersed within the cortical layer of a mitotic HeLa cell, shown for both time-forward δ_c^F (a) and time-reverse δ_c^R (b) directions. (c), (d) Corresponding residual time series ζ^F and ζ^R obtained from either signal by subtracting the autoregression model obtained by fitting the time-reverse tip fluctuations. (e) The autocorrelation function for the measured time-forward AFM tip fluctuations (solid black curve), along with the autocorrelation function of the whitened residual signals ζ^F (x) and ζ^R (°). (f) Normalized empirical probabilities for the residual forward $p(\zeta^F)$ and the residual reverse $p(\zeta^R)$ time series corresponding to the observation time $t = 400$ s. (g) Time irreversibility measure $\hat{\sigma}$ obtained as a function of observation time for control (purple circles) and pharmacologically treated conditions: latrunculin-A (green diamonds) and (-)blebbistatin (yellow triangles). The estimated $\hat{\sigma}(t = 400$ s) differs significantly between the control and the latrunculin-A or blebbistatin-treated cells. This is supported by t -test results, yielding p values of 0.047 and 0.029, respectively. For each cell, the total observation time was 400 s at a sampling rate of $f_s = 100$ Hz.

whitened signal behaves as an independent and identically distributed process; see Fig. 3(e). In Fig. 3(f), we depict the normalized probability distributions $p(\zeta^F)$ and $p(\zeta^R)$ of the obtained residual time series for the observation time $t = 400$ s which are then used in the calculation of the KLD and $\hat{\sigma}$; see Eqs. (8) and (9) in Sec. VI. The determined irreversibility measures $\hat{\sigma}$ calculated for different observation times averaged over all cells are plotted in Fig. 3(g). Similar to Ref. [7], we find that $\hat{\sigma}$ decreases with observation time and acquires an approximately constant value for $t \geq 300$ s for all conditions. Notably, the irreversibility measure $\hat{\sigma}$ decreases for both inhibition treatments including latrunculin-treated cells. We conclude that, upon turnover inhibition (latrunculin-A treatment), irreversibility and effective temperature also show different trends.

Following an alternative approach by Ghosal and Bisker [9], we also tested a second measure of time irreversibility EPR_{WTD} , which is derived by spatially coarse-graining the experimental trajectories into discrete states and quantifying the asymmetry in their waiting time distributions (see Fig. S12 and Supplemental Material Sec. E [24]). Consistent with the trends observed for $\hat{\sigma}$, we find that EPR_{WTD} decreases for both inhibition treatments compared to control cells, provided a sufficiently large number of discrete states are used.

V. ASYMMETRIC SPIKES AND THERMAL-LIKE NOISE HAVE AN ANTAGONISTIC EFFECT ON TIME IRREVERSIBILITY

To better understand the inhibition-induced trend of the time irreversibility measure $\hat{\sigma}$ in our experimental data, we modeled the dynamics of the AFM tip embedded in the viscoelastic actomyosin cortex as an overdamped active Brownian probe subject to active forces. The mechanical response of the cortex is modeled by a viscoelastic Jeffrey's fluid; see Fig. 4(a) and Supplemental Material Sec. C [24]. Furthermore, the mechanical response of the cantilever is captured by a Kelvin-Voigt-type response as characterized by experiments; see Fig. 4(a). Activity is incorporated by scaling thermal forces by a factor $f_{\text{act}} \geq 1$; see Supplemental Material Sec. C [24]. Additionally, we introduce active forces F_{cd} arising from the diffusion of the caging harmonic potential to account for the slow meandering of the AFM tip around its zero position observed in experimental trajectories. Lastly, we incorporate burstlike active forces F_{burst} , characterized by randomly occurring, short-lived force spikes—either symmetric downward or asymmetric upward—closely resembling the active force bursts observed in experiments [see Supplemental Material Sec. C and Eqs. (S1)–(S4) [24]].

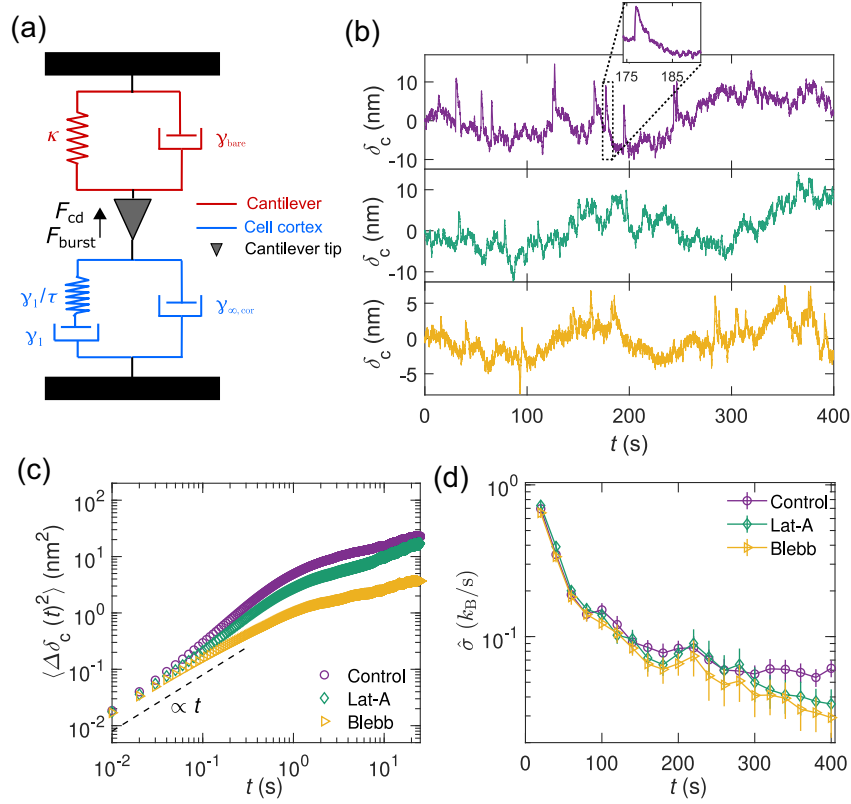


FIG. 4. Minimal model conceptualizing trends in time irreversibility and thermal noise. (a) Schematic illustration of the mechanical environment of the cantilever tip when embedded in the cellular cortex. While the cantilever spring is captured by a Kelvin-Voigt model with cantilever spring constant κ and friction coefficient γ_{bare} , the cortex material is modeled as viscoelastic Jeffreys fluid. (b) Representative numerically simulated trajectories based on the model described by Eqs. (S1)–(S4) [24], corresponding to the control case (top), latrunculin-treated cells (middle), and blebbistatin-treated cells (bottom), respectively. (c) Mean-squared displacements $\langle \Delta \delta_c(t)^2 \rangle$ obtained by averaging over 15 independent trajectories for parameter sets mimicking the control (purple), latrunculin-treatment cells (middle), and blebbistatin-treated cells (bottom). (d) Time irreversibility measure $\hat{\sigma}$ obtained using the Kullback-Leibler divergence approach from 15 independent numerically simulated trajectories of the AFM tip using the model described in (a). The simulated Langevin equation and corresponding parameters are given in Supplemental Material Sec. C [24].

Exemplary simulation trajectories mimicking the three measurement conditions (purple, control; green, actin turnover inhibition; yellow, motor protein inhibition) are shown in Figs. 4(b) and S8 [24]. Parameters were adjusted such that all salient features of experimental trajectories were reproduced. In particular, force spike and dip amplitudes were reduced in the treatment conditions (green and yellow trajectories), consistent with experimental observations [see Figs. S1(c), S1(f), and S3 [24]]. Acknowledging the effect of cortex activity, thermal noise is increased by a frequency-dependent upscaling of thermal noise according to our measurements of effective temperature for the different conditions; see Supplemental Material Sec. C and Fig. S13 [24]. Furthermore, cortex mechanics parameters were varied according to rheological measurements under different experimental conditions (see Sec. C [24]). By computing 15 trajectories over 400 s for each condition, we obtained the MSD and irreversibility measure $\hat{\sigma}$; see Figs. 4(c) and 4(d). Our results show that the MSD is lower in simulations mimicking

inhibition treatments with a stronger reduction for motor protein inhibition [see Fig. 4(c)], similar to experimental results; see Fig. 2(c). Furthermore, $\hat{\sigma}$ is reduced for both turnover and motor inhibition compared to control; see Fig. 4(d).

Performing a systematic analysis, we identify the thermal noise scaling factor f_{act} and force spike amplitude $\mathcal{A}_u$ as key parameters regulating $\hat{\sigma}$. In particular, we find that increasing the thermal noise amplitude via f_{act} decreases $\hat{\sigma}$ but increases the MSD; see Figs. S9(a) and S9(d) [24]. Conversely, upscaling asymmetric force spikes through the amplitude $\mathcal{A}_u$ increases both $\hat{\sigma}$ and the MSD; see Figs. S9(b) and S9(e) [24]. This analysis suggests that, for turnover inhibition, reduction of $\hat{\sigma}$ results from diminished force spike amplitudes. For motor inhibition, reduction of time irreversibility $\hat{\sigma}$ is primarily due to a significant drop in force spike amplitude, despite lower thermal noise. Interestingly, we see that, when both force spike amplitude $\mathcal{A}_u$ and thermal noise amplitude f_{act} are upscaled jointly, $\hat{\sigma}$ shows still a net increase; see Figs. S9(c) and S9(f) [24].

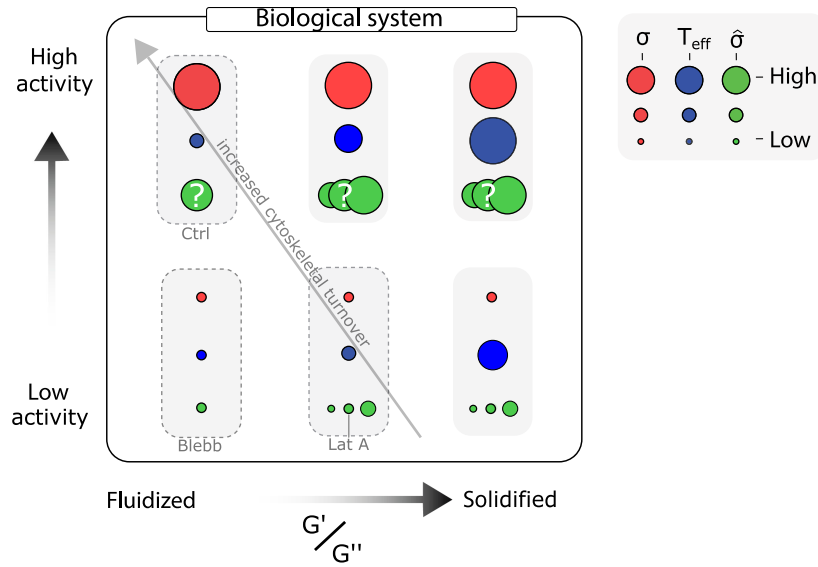


FIG. 5. Schematic illustrating the dependence of nonequilibrium quantifiers—entropy production rate (σ) associated with FDT violation, effective temperature (T_{eff}), and time irreversibility ($\hat{\delta}$)—on mechanical properties and biological activity (ATP consumption). Both entropy production σ and T_{eff} increase with activity (y axis), but T_{eff} also depends on the mechanical properties of the cell or tissue (x axis): Greater solidity tends to promote larger fluctuations and, thus, larger T_{eff} . For time irreversibility $\hat{\delta}$, we propose that it increases with activity in living systems due to a higher occurrence of time-asymmetric spikes arising from excitable dynamics or cycles of material failure and repair. Since this relationship has not been fully confirmed, we mark the corresponding trend with a question mark. Moreover, changes in mechanical properties may affect time irreversibility $\hat{\delta}$ in both directions: an increase in fluctuation amplitudes could raise the likelihood of failure-repair cycles, thereby enhancing the frequency of time-irreversible motifs in the dynamics. Conversely, a mere increase in thermal-like noise might suppress the relative contribution of structured, asymmetric patterns and, thus, reduce $\hat{\delta}$.

Therefore, a coupled upregulation of all active force contributions leads to enhanced time irreversibility.

Our theoretical analysis suggests that, *a priori*, there is no reason why time irreversibility and effective temperature should, in general, exhibit similar trends upon perturbations as opposite trends of these two quantities can be induced through, e.g., a change in the thermal noise amplitude. However, in living systems, a coupling between thermal noise and time-asymmetric deflection spikes might be frequently implemented through an activity-regulated scaling of both noise levels and the frequency or amplitude of time-asymmetric deflection spikes triggered by (i) threshold-gated excitable dynamics (see, e.g., Refs. [45–47]) or (ii) events of material failure and subsequent regulated repair processes [37,48].

In the case of the here-investigated actin cortex, this coupling may arise through the following plausible biological mechanism: Increased thermal-like noise raises the probability of tension-gated calcium channels opening in the plasma membrane, triggering a transient calcium spike and concerted activation of myosin motors [49]. The induced force spike equilibrates over time due to a gradual myosin deactivation and restoration of preceding myosin activity levels [40]. Thereby, a time-asymmetric spike in cortical tension would be generated whose frequency is scaled by the noise amplitude.

In line with this reasoning, we find that, when we modify our model so that force spikes are no longer triggered at a fixed frequency but instead occur when stochastic forces exceed a threshold (see Sec. VI), increasing the thermal noise amplitude no longer reduces time irreversibility but increases it due to the rising spike frequency (see Supplemental Material Fig. S10 [24]).

A similar trend can be observed in the excitable dynamics of membrane potentials in nerve cells—a key regulator of information transmission in the nervous system. Specifically, using the Fitzhugh-Nagumo model [50], we reproduce that increasing noise levels lead to a higher frequency of time-asymmetric spikes in membrane potential and, consequently, greater time irreversibility in the overall membrane potential dynamics; see Fig. S11 [24].

However, the coupling between thermal noise strength (effective temperature) and time irreversibility can be disrupted if material properties change concurrently—for example, through alterations in cytoskeletal polymerization rates (see Fig. 5).

VI. DISCUSSION

In this study, we investigated active position fluctuations $\delta_c = \Delta F/\kappa$ of a soft AFM cantilever tip embedded within

the actin cortex of mitotic human cells. The actin cortex is a manifestation of the actin cytoskeleton—a biological material that not only provides the structural scaffold of eukaryotic cells, but also serves as the core contractile element in muscle tissue. Understanding its non-equilibrium properties is, therefore, fundamental to cell and tissue mechanics across diverse living organisms. Our work represents the first experimental realization of AFM-based measurements of active fluctuations. Unlike previous studies in interphase cells (see, e.g., Refs. [16,17,35,51]), we observed distinct active force bursts in the AFM readout—corresponding to abrupt upward or downward displacements of the AFM probe tip. We conjecture that these bursts arise from pulses of synchronized myosin motor activation (position spikes) and sudden cortical material failure (position dips). Active bursts significantly enhanced the MSD of the cantilever tip, leading to a transition from diffusive to superdiffusive behavior, indicating the presence of active fluctuations in addition to passive thermal fluctuations. Quantifying the power spectral density and the response function of the cantilever tip position, we find that the effective temperature of cantilever fluctuations is significantly increased by an activity factor of up to approximately 100 for timescales $\gtrsim 1$ s in agreement with earlier results from tweezer measurements [17]. We observed that force spikes showed an asymmetric shape with a rapid rise and a slower exponential relaxation with a characteristic time of $\tau \approx 1.5$ s. Correspondingly, we find a nonvanishing time-reversal asymmetry measure $\hat{\sigma}$ derived from the KLD between the whitened forward and time-reversed signal.

To probe the influence of biological activity on activity signatures in the cantilever tip fluctuations, we inhibited two active processes in the actin cortex: (i) the dynamic turnover of the cortical actin matrix and (ii) the activity of cortex-associated motor proteins. While turnover inhibition resulted in only a minor change in the effective temperature of fluctuations, motor protein inhibition led to a dramatic decrease, underscoring the crucial role of motor proteins in driving active fluctuations. Counterintuitively, despite exhibiting similar values of $T_{\text{eff}}(f)$ as the control, turnover-inhibited cells displayed a lower entropy production rate σ associated with the corresponding FDT violation as inferred from the Harada-Sasa equality. This can be attributed to the more solidlike elastic nature of the cortex in turnover-inhibited cells, leading to reduced energy dissipation for a given noise level. Therefore, consistently, we observe that entropy production decreases for both types of activity reduction. However, this trend is not reflected in both cases by a corresponding change in effective temperature; see Fig. 5.

Finally, we find a reduced time irreversibility $\hat{\sigma}$ in both types of activity-inhibited cells. Thus, interestingly, the changes of the time irreversibility measure $\hat{\sigma}$ agree qualitatively with the changes of entropy production σ associated

with the FDT violation of tip fluctuations. To rationalize these trends in time irreversibility, we reproduced experimental tip trajectories in simulations of an overdamped Brownian probe embedded in a non-Markovian viscoelastic medium subjected to active force bursts and upscaled Brownian motion. Our simulations indicate that time irreversibility in activity-inhibited cells is reduced due to a decrease in force spike amplitudes. Interestingly, our simulations also show that a mere upscaling of thermal noise (and, correspondingly, of effective temperature) leads to a reduction of the time irreversibility.

In conclusion, our study demonstrates that different nonequilibrium measures such as effective temperature T_{eff} , entropy production σ , and time irreversibility $\hat{\sigma}$ can exhibit contrasting behaviors in active living systems, depending on the nature of the active processes and the properties of the surrounding biopolymer scaffold; see Fig. 5. In particular, we find that entropy production associated with FDT violation and effective temperature may display opposing trends when increased activity fluidizes the material or, conversely, when reduced activity leads to solidification; see Fig. 5. Although activity is often associated with the emergence of active stresses [52] and stress-induced stiffening [53–55], several studies have reported that enhanced active stress can increase the material's loss tangent and fluidization [34,56–59]. Furthermore, our results put forward that reducing polymerization rates of cytoskeletal scaffolds is a major mechanism for simultaneously decreasing active stresses and promoting solidification by extending the material's turnover and relaxation times. In summary, our findings challenge the validity of effective temperature as a reliable proxy for the distance from thermal equilibrium in cases where activity modulates the mechanical state and fluidity of the material.

Furthermore, while our experimental measurements reveal similar trends for FDT-violation-associated entropy production σ and time irreversibility $\hat{\sigma}$, our simulations suggest that certain types of perturbations can decouple these quantities, affecting them in opposite ways. Notably, probe dynamics in an active medium may exhibit elevated activity without corresponding time irreversibility—for example, when activity is expressed solely as enhanced thermal fluctuations, reflected in an increased effective temperature. In living systems, time irreversibility is often introduced into a probe trajectory through inherently time-asymmetric, excitable dynamics. Examples include fluctuations in forces or material properties regulated by membrane action potentials, intracellular calcium spikes [45–47], or cycles of cytoskeletal failure and repair [37,48], all of which can introduce directional motifs into the dynamics. It remains an open and intriguing question whether a fundamental principle underlies the coupling between entropy production and time irreversibility in living systems—for instance, through an activity-dependent regulation of excitable event frequency.

VII. MATERIALS AND METHODS

A. Cell culture

We cultured the HeLa Kyoto cells in the growth medium Dulbecco's Modified Eagle Medium (DMEM) (No. 31966-021, Life Technologies) in combination with 10% v/v fetal bovine serum (FBS) (No. 10270106, Life Technologies) and 1% v/v penicillin/streptomycin (No. 15140122, Life Technologies) at $T = 37^\circ\text{C}$ and 5% CO_2 . The splitting was performed every 2–3 days upon reaching a confluency of 60%–80%. Around 10000 cells were seeded a day prior to the experiment at the center of a 3.5 cm glass Petri dish. The seeding area was confined within a silicon chamber with a media volume of 250 μL ensuring precise spatial localization of the cells in the center of the dish. On the measurement day, the silicon chamber was removed, and the growth medium was replaced with 2mL CO_2 -independent DMEM (No. 12800-017, Invitrogen) containing 4 mM NaHCO_3 , buffered with 20 mM HEPES/NaOH at $\text{pH} = 7.2$, and enriched with 10% FBS. Additionally, the medium was supplemented with 2 μM S-trityl-L-cysteine (No. 164739, Sigma) which inhibits kinesin Eg 5 arresting cells in mitosis. Cells were then kept in an incubator maintained at $T = 37^\circ\text{C}$ without CO_2 for 2–3 hours until mitotic cells were visibly enriched.

For measurement of drug-treated cells, pharmacological agents (–) – blebbistatin (10 μM , No. B0560-1MG, Sigma) or latrunculin-A (25nM, No. AG-CN2-0027, AdipoGen Life Sciences) were added to the medium at least 15 min prior to measurements.

B. Atomic force microscopy measurements

The experiments were performed with a Zeiss Axiovert inverted light microscope combined with an AFM Nanowizard 4XP (JPK Instruments) using a $20 \times / 0.8$ air objective. The Petri dish containing cells was maintained at 37°C using a Petri dish heater (JPK Instruments). All measurements were performed using triangular MLCT-D cantilevers (Bruker) with nominal spring constant $\kappa = 0.03\text{N/m}$. On each day of the measurement, the spring constant κ of the cantilever was separately determined using the built-in software (JPK Instruments) which exploits the power spectral density of the thermal fluctuations.

1. Measuring active fluctuations and rheology

We chose mitotic HeLa cells for this study as previous work showed a dominant effect of the actin cortex upon mechanical deformation with an AFM cantilever [21].

First, a cell in mitotic arrest was chosen. Then, an approach was performed on top of this cell with the tip touching approximately the center of the projected cell area. After cantilever retraction, the cantilever was extended with a speed of 2 $\mu\text{m/s}$ up to a peak force of 1 nN. Subsequently, the piezo-controlled cantilever hinge

height was kept constant for a time interval of 500 s. After the initial force peak, the tip deflection was allowed to equilibrate for 100 s. Then, the measurement was performed for further 400s where the time t , hinge height h , and the tip deflection F was recorded for later analysis of tip height fluctuations $\delta_c = F/\kappa$. Depending on the measurement, the sampling frequency was kept between 100 and 1000 Hz.

For rheology measurements, h was varied sinusoidally across a range of frequencies from 0.02 to 10 Hz, with an amplitude of 200 nm. Figure S4 [24] presents a typical time evolution of the oscillating cantilever hinge height h and the corresponding tip position oscillations δ_c for frequencies between 0.05 and 10 Hz.

2. Mean-squared displacements

We first quantified the active nature of AFM tip fluctuations embedded within the cortex by the mean-squared displacement $\langle \Delta \delta_c(t)^2 \rangle = \langle [\delta_c(t + t_0) - \delta_c(t_0)]^2 \rangle$ which are plotted in Fig. 2(a). In order to calculate $\langle \Delta \delta_c(t)^2 \rangle$, a time average over t_0 is first computed for a given trajectory and then ensemble average is performed over the different realizations on cells N for each case.

3. Microrheology

Following Refs. [60,61], we compute the frequency-dependent complex elastic shear modulus $G_{\text{eff}}^*(f)$ of the cortical layer from the externally imposed sinusoidal oscillations of different frequencies of the tip motion (see Fig. S6 [24]) using

$$G_{\text{eff}}^*(f) = \frac{\sqrt{2}}{6 \tan(\beta) \delta_0} \frac{\hat{F}(f)}{\hat{\delta}_h(f)} \exp(i\Delta\varphi), \quad (4)$$

where $\hat{F}(f)$ and $\hat{\delta}_h(f)$ are the fitted amplitudes of the sinusoidal force signal and the indentation depth $\delta_h(t) = -(h(t) + \delta_c(t))$ with $h(t)$ the piezo height of the AFM. Here, $\beta = 17.5^\circ$ is the half-angle to edge of the pyramidal tip of the MLCT cantilever and δ_0 is the average indentation depth within the cortex as determined from our measurement separately on each cell. We find that δ_0 typically adopts values of approximately 1 μm . Beyond contributions from viscoelastic stresses, measured force amplitudes are expected to show contributions that stem from active stresses coupling to geometrical changes; see Ref. [53].

Given the molecular turnover in the cortex, we can infer that active stress contributions dominate the force signal at small frequencies [53]. Therefore, we estimate the activity-corrected complex elastic shear modulus $G^*(f)$ by $G^*(f) = G_{\text{eff}}^*(f) - G'_{\text{eff}}(0.02 \text{ Hz})$; see Fig. S5 [24].

4. Fluctuation dissipation theorem

The FDT provides a direct link between mechanical properties captured by the response function $\chi(f)$ and the

strength of fluctuations in the linear regime. Its violation indisputably confirms the deviations from equilibrium. Mathematically, it relates the power spectral density $C(f)$ of the tip fluctuations $\delta_c(t)$ to the imaginary part of the response function $\chi''(f)$ as

$$\frac{2\pi C(f)}{2k_B T} = \chi''(f). \quad (5)$$

In our experiments, $C(f)$ was obtained from the fast Fourier transformation of the measured tip fluctuations $\delta_c(t)$ using MATLAB as

$$\begin{aligned} \tilde{\delta}(f) &= \text{FFT}[\delta_c(t)], \\ C(f) &= \frac{\tilde{\delta}(f)\tilde{\delta}^*(f)}{f_s p}, \end{aligned} \quad (6)$$

where $\tilde{\delta}^*(f)$ is the complex conjugate of $\tilde{\delta}(f)$, p is the number of measurement points in δ_c , and f_s is the sampling rate. The imaginary part of the response function $\chi''(f)$ was determined by performing sinusoidal indentations in the cell cortex at various frequencies f with amplitude 200 nm and measuring the resultant linear response $\chi(f) = \hat{\delta}_h(f)/\kappa \hat{h}(f)$. We compute the effective temperature as $T_{\text{eff}}(f) = \pi C(f)/k_B T \chi''(f)$ from the experimental data; we performed binning of the response measured from $C(f)$ over a frequency window with width $\epsilon = 0.01$ Hz, i.e., $f \pm \epsilon/2$.

C. Calculation of the Kullback-Leibler divergence

Following Roldán *et al.* [7], we determine the irreversibility measure $\hat{\sigma}$ by calculating the KLD of the measured stochastic time series $\delta_c(t)$ of the AFM tip position embedded within the cortex for both the treated and untreated cases. For this purpose, we obtained from the experimentally measured time series $\delta_c^F(t)$, the time-reversed series $\delta_c^R(t)$ by inverting the time index, as demonstrated in Figs. 3(a) and 3(b). An autoregression model $\text{AR}(m)$ of the order of $m = 25$ is then obtained by fitting the reverse time series using the MATLAB “arfit” package developed by Schneider and Neumaier [62]. Using the obtained $\text{AR}(25)$ model, the residual time series for both the forward and the corresponding reverse time series are computed as

$$\xi^{\text{F,R}} = \begin{cases} \delta_c^i & \text{if } i \leq m, \\ \delta_c^i - \sum_{j=1}^m a_j \delta_c^{i-j} & \text{if } i \geq m, \end{cases} \quad (7)$$

where δ_c^i is the i th element of the forward or reverse time series $\delta_c^{\text{F,R}}(t)$ while a_j is the j th coefficient of the AR model; see Figs. 3(c) and 3(d). The residual time series corresponding to the forward and reverse trajectories are plotted in Figs. 3(c) and 3(d). This process is called

whitening, as it removes the time correlations present in the time series, rendering the resultant time series $\xi_c^{\text{F,R}}(t)$ as an independent and identically distributed process. We choose $m = 25$ for analysis, as it effectively removes all the time correlations present in the measured trajectories. This is demonstrated by the autocorrelation functions for both the residual forward $\xi^F(t)$ and residual reverse $\xi^R(t)$ time series in Fig. 3(e). To ensure effective whitening (i.e., removal of temporal autocorrelations), we systematically evaluated AR models of increasing order from 2 to 25; see Supplemental Material Fig. S7 [24]. We observed that the autocorrelation functions of the whitened signals began to converge around order 10, indicating that most temporal correlations were already removed by that point. We selected order 25 as a conservative choice to ensure robust whitening across all datasets while still maintaining reasonable computational efficiency.

In Fig. 3(f), we depict the normalized probabilities $p(\xi^F)$ and $p(\xi^R)$ which were computed considering 50 bins spanning from the minimum to the maximum values of the residual series. The irreversibility measure $\hat{\sigma}$ is calculated as

$$\hat{\sigma} \geq k_B f_s D[p(\xi^F)||p(\xi^R)], \quad (8)$$

where

$$D = \gamma \sum_i p(\xi_i^F) \ln \left(\frac{p(\xi_i^F)}{p(\xi_i^R)} \right) \quad (9)$$

is the KLD. The prefactor γ accounts for the statistical bias of the KLD estimate [7]. It is obtained using the Kolmogorov-Smirnov (KS) test under the null hypothesis $H_0: p(\xi^F) = p(\xi^R)$, which yields a p value p_{KS} . The prefactor γ is defined as $\gamma = 1 - p_{\text{KS}}$.

Corresponding results for $\hat{\sigma}$ are found in Fig. 3(g). Remarkably, determined $\hat{\sigma}$ values were comparable to those observed in the cortex of starfish oocytes [63].

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N. N. performed the experiments and data analysis. N. N. and E. F.-F. designed the experiments. N. N. and E. F.-F. wrote the manuscript.

The authors declare no competing interests.

DATA AVAILABILITY

The data that support the findings of this article are openly available [64]; embargo periods may apply.

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