Scale-free aggregation and interface fluctuations of cancer clusters in cancer-endothelial cell mixtures: From the dilute state to confluent monolayer

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We experimentally investigate the multiscale dynamical and scaling behaviors of the morphology, motion, and area distribution of segregated cancer clusters of proliferating cancer-endothelial cell mixtures, from the initial randomly distributed dilute state to the jammed confluent monolayer state. Cancer cells (CCs) have higher motilities than endothelial cells (ECs) and weaker CC-EC and CC-CC cohesive couplings than EC-EC coupling. It is found that, with increasing waiting time t_w , CCs proliferate, move, and aggregate into clusters with fractal cluster boundaries, increasing averaged cluster area, and the gradual transition to the self-similar power-law distribution of single cluster areas. In CC clusters, multiscale turbulentlike motion and the interaction with surrounding ECs are the keys for self-similar multiscale spatial fluctuations of the velocities and fractal structure of CC cluster boundaries. The larger CC cluster allows stronger longer-length fluctuations. It causes the increasing fractal dimension and scaling exponents of the relative motion of CC cluster boundaries, which can be suppressed by proliferation-induced crowding and dynamical slowing down.

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Fluctuating interfaces ubiquitously occur in various nonequilibrium media [1]. The interplay of different coherences generated by different mutual couplings on the two sides of the interface, and the disorder generated by passive/active forces, stochastic agitations, or mass inputs, could generate self-similar multiscale spatiotemporal interface fluctuations [1]. Solid-liquid fronts in melting or solidification [1–3], paper burning or wetting fronts [1,4,5], fronts of diffusion-limited aggregations [1,6], and invading interfaces of mobile bacteria into demobilized bacteria [7] are a few good examples.

For passive or active *binary mixtures*, the same type of species could segregate into clusters [8–17]. For example, for binary mixtures at the continuous limit, clusters with smooth boundaries governed by surface tension can be formed through spinodal decomposition [8,12]. However, the generic dynamical behaviors of aggregation and fluctuating cluster boundaries of binary mixtures down to the *microscopic* level, especially for *active* cell systems, remain elusive.

The cell system is a coupled active system exhibiting motions over a wide range of scales, which are important in many biological processes such as embryogenesis [18], tumorigenesis [19], and cancer metastasis [20–22]. Comparing with coupled passive systems, the additional self-propelled motion, unfrozen proliferation, deformable cell boundary, etc., enrich the structural and dynamical behaviors [23–33]. For example, a proliferation-induced liquid-glassy transition, and collective

motion in the form of multiscale swirls, turbulence, follow the leader streaming, and chain-type collective migration, have been demonstrated in *monotype* cell systems [23–28]. The fractal-like distribution of cell nuclei in the arrested epithelial cell clusters with highly suppressed motility by tuning growth factors was also reported [33].

For cell mixtures, the behaviors of structure and motion can be further enriched [10–17,34]. The segregation of the same type of cells into clusters due to the different motilities and different cell-cell couplings of the two different types of cells is a special feature. Numerous works reported that tumors exhibit scale-free fractal patterns and fractal boundaries. However, they were measured from static histological images of stained tissues without addressing dynamical behaviors [35–37]. Past studies on the dynamical behaviors of other binary cell mixtures have mainly focused on finding the temporal evolutions of the sizes of dominant segregated clusters [10–14] and the degrees of segregation [15–17], by tuning cell motility and coupling. The recent experimental work on the invasion of a small fraction of more mobile cancer cells (CCs) into the *confluent* monolayer endothelial cells (ECs) demonstrated that the aggregation of CCs into a larger cluster enhances turbulentlike CC motion and rejuvenates the jammed motion of the surrounding ECs induced by proliferation [23].

Nevertheless, the multiscale structural and dynamical behaviors of aggregation and fluctuating cluster boundaries, and their correlation with turbulentlike cell motions of the proliferating monolayer binary cell mixture, from the *dilute* state with similar number fractions of the two types of cells with different motilities, remain unexplored. Here, using the CC-EC mixture as an example, the above intriguing issues are experimentally unraveled. We demonstrate the gradual aggregation of individual CCs and small CC clusters into larger

FIG. 1. (a) Phase contrast images of the CC-EC mixture at $t_w = 0, 10, 30,$ and 50 h, respectively. CCs have brighter cell boundaries. (b) Spatiotemporal distribution of CC clusters from the same region as that of (a). (c) Probability distribution functions P_A of the CC cluster area A at different t_w , showing the stretching of the descending tails to the scale-free power-law decay with the scaling exponent equaling -1.3 , as t_w increases. (d) MSDs of CCs and ECs at different t_w from the Lagrangian trajectory of each element, with the numbers indicating the scaling exponents of the reference straight gray line. Inset: Temporal evolutions of cell density (cell number divided by image area) and MSD over the 20-min interval of CCs and ECs. Straight lines between data points in the inset are used as guides for the eye.

CC clusters, associated with the transition to the scale-free CC cluster size distribution, and illustrate how the turbulent CC motions lead to the observed scale-free fluctuations of the velocity and positions of CC boundaries, associated with the dynamical slowing down through proliferation-induced crowding.

We use bone marrow metastasis nasopharyngeal carcinoma cells (CCs) and human umbilical vein endothelial cells (ECs) [38]. The CC has a higher motility than that of the EC. The strengths of mutual cell-cell adhesion from strong to weak follow the order: EC-EC (strong VE-cadherin junctions), CC-CC (weak E-cadherin junctions), and EC-CC (no junctions) [38]. The initial CC/EC number ratio is 1.5 (the initial packing densities of CCs and ECs are 88 and 58 cell/ mm^2 , respectively), for easily forming large CC clusters separated by surrounding ECs [38]. The waiting time t_w is set to 0 when randomly distributed CCs and ECs simultaneously land on a collagen-coated substrate [Fig. 1(a)]. The spatiotemporal dynamics of the CC-EC monolayer is recorded by a time-lapse phase contrast microscope. The velocity fields are obtained using particle image velocimetry (PIV) [39]. More experimental details of cell incubation, PIV, CC cluster boundary identification and Lagrangian trajectory tracking can be found in the Supplemental Material (SM) [38].

Figure 1(a) shows the phase contrast images of the CC-EC mixture at $t_w = 0, 10, 30,$ and 50 h. CCs have bright cell boundaries. Figure 1(b) shows the temporal evolution of CC clusters in the xyt space (also see Video 1 [38]). At $t_w = 0$ h, the dilute CCs and ECs are mainly circular. After attaching to the substrate, cells spread and CCs gradually aggregate into clusters with irregular boundaries. After 30 h, a confluent monolayer is formed. As demonstrated in Figs. 1(a) and 1(b), continuing cell proliferation [see the increasing cell density with t_w in the inset of Fig. 1(d)] and aggregation increase the averaged CC cluster size. The double-logarithmic

plots of P_A , the probability distribution function of the CC cluster area A , in Fig. 1(c) show the gradual stretching of the descending tails to the scale-free power-law decay with increasing t_w .

Cells move in the above process. Figure 1(d) shows the curves of CC and EC mean-square displacements (MSDs) over a time interval τ from the Lagrangian trajectory of each element in the two types of cells (see SM [38] for the trajectory construction), starting in 1-h intervals after $t_w = 10, 30,$ and 50 h. All the curves with scaling exponents greater than 1 manifest superdiffusion. At each t_w , the larger MSD with the larger scaling exponent for CCs indicates the faster and more persistent motion than that of ECs. Moreover, MSD curves are upward shifted before $t_w = 30$ h and downward shifted after $t_w = 30$ h. The former can be attributed to the enhanced cooperative motion through the increasing mutual coupling caused by the proliferation and aggregation into larger clusters. The latter indicates the dynamical slowing down due to proliferation-induced crowding. They can also be clearly manifested by the inset of Fig. 1(d), showing the temporal dependence of MSDs of CCs and ECs over a 20-min interval, respectively. The speeding-up/slowing-down transitions for CCs and ECs both occur around $t_w = 30$ h, the time for cells forming a confluent cell monolayer.

CCs aggregate into clusters with irregular boundaries (Videos 1 and 2 [38]). Figure 2(a) shows the evolution of CC cluster boundaries, enlarged from the boxed region of Fig. 1(a) at $t_w = 10, 30,$ and 50 h. The gray areas are CC clusters at t_w and the blue solid lines are CC cluster boundaries 1 h after t_w . The irregular CC cluster boundaries still fluctuate spatiotemporally even after forming the confluent cell monolayer for $t_w \geq 30$ h.

CC cluster boundaries exhibit a fractal structure. It can be evidenced by the power-law increase of $\langle N(r) \rangle$ with r in Fig. 2(b), where $N(r)$ is the number of pairs of points

FIG. 2. (a) Evolutions of CC clusters at $t_w = 10, 30$, and 50 h, enlarged from the boxed region of Fig. 1(a). The gray areas are CC clusters at each t_w and the blue solid lines are CC cluster boundaries 1 h after t_w . (b) Averaged number of pairs of points $\langle N(r) \rangle$ with separation r on the boundaries of all clusters at different t_w . The numbers by the gray lines correspond to the scaling exponents (fractal dimensions). (c) Temporal evolutions of the fractal dimension D of the boundaries of all clusters and averaged cluster area $\langle A \rangle$. (d) Averaged fractal dimension D_A of the boundaries of the single CC cluster with area A . (e) Shape index p of the single CC cluster boundary vs CC cluster area A .

with a separation smaller than r on the boundaries of all CC clusters in each image, and the average is taken over 1-h images after a fixed t_w . Namely, the structural fluctuation is statistically similar to itself on magnification over a finite range of length scales. The slope of each double-logarithmic curve corresponds to the fractal (correlation) dimension D , which also quantifies the effective dimensions and degrees of complexity of the irregular CC boundary [1].

Each single CC cluster boundary is also fractal (Fig. S4 [38]). Figure 2(d) shows D_A , the fractal (correlation) dimension of the boundary of the *single* CC cluster with area A , averaged over all those clusters from the 5-h images after each t_w . The curves of D_A all increase with A . The increasing interface complexity can be contributed by the stronger fluctuations of the longer scales in the larger CC cluster, and further supported by Fig. 2(e) showing the increasing shape index p with cluster area A . Here, p is defined as the averaged ratio of the CC cluster boundary length to the square root of CC cluster area [25], which also measures interface irregularity. D , D_A , and p show faster descents after $t_w \geq 30$ h.

We further characterize the spatial scaling behavior of velocity fluctuations along the boundary of the *single* cluster with area A by measuring the mean-square relative velocity, $\text{MSRV} = \langle |\delta \mathbf{V}(r)|^2 \rangle$, where $\delta \mathbf{V}(r) = \mathbf{V}_i - \mathbf{V}_j$ is the velocity difference of two elements i and j with separation r on a CC cluster boundary. The average is taken over all the single CC clusters with the same area A of the images within a 5-h time interval. As shown in Fig. 3(c), all the MSRV curves exhibit a power-law increase with r until leveling off around $r = 50\ \mu\text{m}$ (i.e., the velocity correlation length shown in Fig. S6 [38]). The scaling exponent α and the magnitude of MSRV both slightly increase with increasing A from 2×10^3 to $3 \times 10^4\ \mu\text{m}^2$. With increasing t_w , they first slightly rise and then decrease after $t_w = 30$ h. $\alpha > 1$ implies the superlinear

nonrandom scale-free increase of MSRV with increasing r , contributed by the multiscale turbulent CC motions.

What is the origin of CC clusters with scale-free cluster size distributions and scale-free boundary fluctuations in structure and motion? In continuous passive binary mixtures, segregated patterns exhibit smooth boundaries for minimizing interfacial energy [8,9]. However, in our active binary mixture, the difference in cell motilities and intercellular coupling of CCs and ECs, and the floppy cell boundaries allow richer behaviors in structure and motion. The previous study on the invasion of randomly distributed low concentration CCs into a confluent and dense EC monolayer demonstrated the aggregation-enhanced turbulentlike multiscale CC motion with increasing CC cluster size [23]. This turbulent CC motion is certainly a source for the fluctuating CC cluster boundary.

In this study, starting from the low-density CCs and ECs with 1.5/1 concentration ratio, CCs can aggregate into larger clusters than those in the previous study [23]. Figure 3(a) and the left panel of Fig. 3(b) enlarged from the boxed region of Fig. 3(a) show typical plots of the velocity fields color coded by velocity orientation, where the gray areas represent the areas of CC clusters. The enlarged plot in the right panel of Fig. 3(b) illustrates the trajectories over 1 h, color coded by trajectory length. Video 2 [38], and the power-law decay of the power spectra of temporal evolutions of CC element Lagrangian velocities in Fig. S5 [38] further show scale-free turbulentlike CC motion. The spatial correlation length of Eulerian CC velocity increases from 55 to $60\ \mu\text{m}$ and then decreases to $50\ \mu\text{m}$ with increasing cell coupling as t_w increases (Fig. S6 [38]). It corresponds to the scales of colored patches in the large clusters.

The motion coherence of a single CC cluster, defined as the magnitude of the mean velocity normalized by the mean speed of all the elements in the CC cluster, decreases with

FIG. 3. (a) Velocity fields of the same region as that of Fig. 2(a), color coded by velocity directions at different t_w . The gray regions are CC clusters. The length of the vertical black vector at the lower right corner represents a velocity vector with speed = 2 $\mu\text{m}/\text{min}$. (b) Left: Enlarged velocity field from the boxed region of (a) at $t_w = 50$ h. Right: 1-h element trajectories color coded by the trajectory length L of the same region as the left panel. (c) MSRV vs r , for clusters at $A = 2 \times 10^3$ and 3×10^4 μm^2 and $t_w = 10, 30$, and 50 h. The numbers by the gray lines correspond to the scaling exponents.

increasing cluster area. For the small CC cluster with a size smaller than 50 μm , the high motion coherence [also see the velocity vectors with similar colors (directions) in the first panel of Fig. 3(a)] can make all CCs move together and merge with other CCs to form larger clusters. In the larger CC cluster, more patches with different velocity orientations lead to decreasing motion coherence with increasing A [see Fig. S6(c) [38]].

Note that, for the *monotype* CC system from the dilute state with increasing cell density through proliferation, the self-propelled cell motions suppress CCs from aggregating into clusters with dense cell packing [see Fig. S3(d) and Video 3 [38]]. However, for our binary CC-EC mixture, the weaker CC-EC coupling than CC-CC and EC-EC couplings leads to the easy formation of CC clusters surrounded by more strongly coupled ECs which have a lower motility than that of CCs (see Video 1 [38]). The CC cluster size can increase through the gradual merging of single CCs or CC clusters with different areas. The turbulent CC motion can also cause the splitting of a CC cluster into smaller clusters. The final power-law cluster size distribution in the later stage [Fig. 1(c)] manifests that similar dynamical rules for aggregation are followed over a certain range of scales.

For a nonequilibrium nonlinear extended system, the interplay between the order/coherence generated by mutual interaction, and the disorder excited by the stochastic drives (such as stochastic thermal agitation or self-driving) or the slow drives, can lead to scale-free spatiotemporal fluctuations over a certain range of the control parameter, as experimentally and numerically demonstrated in many self-organized criticality (SOC)-type systems and interface fluctuations in passive biphasic systems [1–5,40]. In the cell system, the self-driven cell motions provide active drives to generate disorder. In our binary cell mixture, in addition to the aggregation of CCs due to the weakest CC-EC coupling, the self-driving of

mobile CCs and weak CC-CC coupling generate scale-free turbulent CC motions in CC clusters. They are the sources to drive the surrounding ECs with low motility and strong EC-EC coupling, and induce boundary fluctuations.

Namely, for the CC cluster boundary, the multiscale spatiotemporal fluctuations are determined by the interplay of CC turbulence due to high CC motility and low CC-CC coupling, and surrounding ECs with low motility and strong EC-EC coupling, which suppress motion and structural rearrangement. Excited by the scale-free turbulentlike motion in the CC clusters, the CC cluster boundary structure affects the forces on the boundary, and in turn provides a feedback to determine the velocity and subsequent structure of the boundary. It is the key for self-consistently sustaining the correlated scale-free fluctuations of the structure and the velocity of the CC cluster boundary.

Through aggregation, the CC cluster with a larger size and more CCs facilitates longer-scale fluctuations. It causes the consistent increases of D_A , p , α (the scaling exponent of MSRV), and β (the absolute value of the scaling exponent of CC velocity spectrum in Fig. S5) with increasing A . Their consistent faster decreases with increasing t_w for $t_w \geq 30$ h reflect the reduced complexities of CC turbulent motion, and boundary fluctuations both in structure and motion, by proliferation-induced crowding and dynamical slowing down. They also evidence the strong correlation of CC motion in the cluster, and the structure and motion of the fluctuating CC cluster boundary.

The above self-similar multiscale behaviors are akin to the scale-free SOC-type behaviors in certain ranges of control parameters of various nonequilibrium nonlinear extended systems under persistent or stochastic drives, such as avalanche cracking, seismic activities, and melting, wetting, and fire burning interfaces [1–5,40]. For binary cell mixtures, to our knowledge, previous works have not addressed the issues sim-

ilar to ours about the multiscale cluster size distributions, the multiscale structural and dynamical behaviors of the cluster interfaces, and their correlations [10–17].

In conclusion, we have investigated the multiscale area, morphology, and dynamical behaviors of CC cluster aggregation, especially demonstrating the scale-free fluctuations in structure and motion of the CC cluster boundary, in the proliferating CC-EC binary mixture from the dilute to the confluent state. The higher CC motility than EC motility and the weaker CC-EC coupling cause CC aggregation into clusters exhibiting turbulentlike motion with a finite correlation length. The gradual merging of single CCs and CC clusters with different sizes and motion coherence causes the transition from the stretched exponential to the scale-free power-law distributions of the cluster area. The interplay of the multiscale CC turbulent motion and the surrounding less mobile and more strongly coupled ECs through the fluctu-

ating boundary, is the key to self-consistently sustain the correlated scale-free multiscale fluctuations of the structure and velocity of CC cluster boundaries. Increasing cluster area facilitates stronger fluctuations with increasing length scale and increases the scaling exponents of velocity as well as position fluctuations of CC cluster boundaries, which can be suppressed by proliferation-induced crowding and dynamical slowing down.

This study paves a way for investigating and understanding the generic dynamical behaviors of aggregation and multi-scale interface fluctuations in various *active* binary systems, an important issue comparing with the widely studied interface fluctuations of various *passive* binary mixtures.

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