

Network Characteristics of Collective Chemosensing

Bo Sun,^{1,*} Guillaume Duclos,² and Howard A. Stone¹

¹*Department of Mechanical and Aerospace Engineering, Princeton University, Princeton, New Jersey 08544, USA*

²*Physico-Chimie Curie, Centre National de la Recherche Scientifique, UMR 168, Institut Curie,
26, rue d'Ulm, F-75248 Paris Cedex 05, France*

(Received 29 November 2012; published 9 April 2013)

The collective chemosensing of nonexcitable mammalian cells involves a biochemical network that features gap junction communications and heterogeneous single cell activities. To understand the integrated multicellular chemosensing, we study the calcium dynamics of micropatterned fibroblast cell colonies in response to adenosine triphosphate (ATP) stimulation. We find that the cross-correlation function between the responses of individual cells decays with topological distance as a power law for large colonies and much faster for smaller colonies. Furthermore, the strongly correlated cell pairs tend to form clusters and are more likely to exceed the percolation threshold. At a given topological distance, the cross-correlations exhibit characteristics of Poisson distributions, which allows us to estimate the unitary conductance of a single gap junction which is in good agreement with direct experimental measurements.

DOI: [10.1103/PhysRevLett.110.158103](https://doi.org/10.1103/PhysRevLett.110.158103)

PACS numbers: 87.18.Hf, 87.18.Mp, 87.18.Nq

Biological systems use self-organized biochemical networks to make decisions in response to environmental cues and, in particular, to chemical stimuli [1]. At the single cell level, for instance, signaling pathways that involve tens of proteins and ribonucleic acids (RNAs) determine cell differentiation [2] and cell cycle [3]. When intercellular signaling is present, these pathways extend to the multicellular scenario where each cell type plays distinct roles to form subnetworks in a given regulatory circuit [4,5]. However, there is an underappreciated aspect of multicellular networks: when many cells of the same type form a colony and sense biochemical perturbations, the single cell responses are coupled by intercellular communications, which in turn generate integrated responses that can involve the entire population. These processes, which we termed collective chemosensing [6], have been studied to date mostly in prokaryotic cells [7].

Upon chemical stimulation, bacteria and eukaryotic cells such as *Dictyostelium discoideum* employ intercellular “quorum sensing,” which leads to positive feedback between the cells and the messenger molecules [8]. This mechanism allows the population behaviors to be characterized as reaction-diffusion systems [9]. However, the corresponding multicellular dynamics of mammalian cells are much different [6]. In this case, the collective response of a cell colony to chemical stimuli features heterogeneous responses of individual cells linked by nearest-neighbor communications. As a result, it is necessary to develop statistical tools to understand the spatial-temporal dynamics underlying the network of collective chemosensing.

In this Letter, we report the self-organized network responses of (nonexcitable) mammalian cells to chemical stimuli. Our experimental system consists of a high density of fibroblast cells growing on two-dimensional substrates. We stimulate the cell colonies using different

concentrations of adenosine triphosphate (ATP), which is a common signaling molecule that orchestrates various multicellular functions such as platelet aggregation [10] and vascular tone [11]. When P2 receptors on the cell membrane recognize ATP molecules, they release the second messenger inositol trisphosphate (IP3), which activates ion channels of the endoplasmic reticulum calcium stores and generates transient calcium oscillations in the cell cytoplasm [12]. We monitor these calcium dynamics using fluorescent indicators. At the same time, cells that are physically in contact form gap junctions through their membranes, which allow intercellular communication by rapidly exchanging small molecules such as Ca^{2+} and IP3 [13]. Although we use fibroblast cells as our model, P2 receptors are expressed almost universally in mammalian tissues [14]. Since similar calcium dynamics takes place in many cell types and regulates crucial physiological functions such as apoptosis [15] and inflammation responses [16], our model system is representative of a large category of biochemical signaling networks.

To explore the underlying statistical principles of collective chemosensing by mammalian cells, we developed a flow device combined with micropatterning of cells (Fig. 1). Briefly, cleaned glass cover slips were coated with a protein-repellent layer (interpenetrated gel of acrylamide and polyethylene glycol) [17]. Then we used photolithography methods and an air plasma treatment to locally etch the protein-repellent gel [18] so that glass cover slips were patterned with antiadhesive gel and plain glass squares. In the last step we grafted proteins (gelatin) onto the plain glass patches. The treated cover slips were then sealed with polydimethylsiloxane (PDMS) channels using plasma bonding [Fig. 1(a)].

NIH 3T3 fibroblast cells were trypsinized and suspended in growth medium (Dulbecco's modified Eagle medium

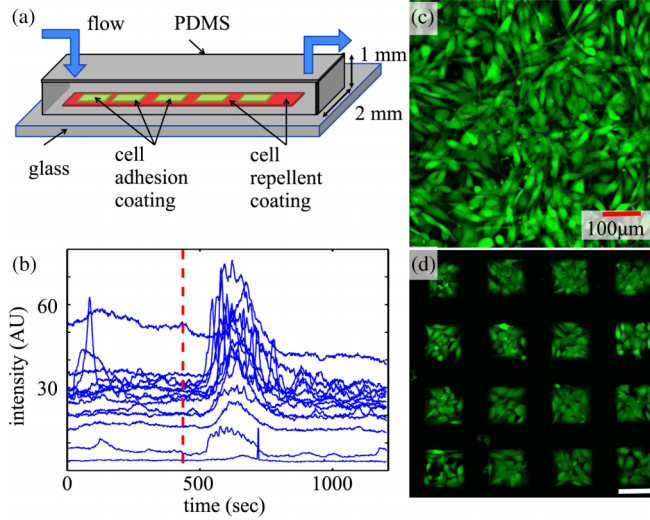


FIG. 1 (color online). The experiment setup. (a) Flow devices were made by sealing micropatterned glass and PDMS channels. By using photolithography, cell repellent (interpenetrated polyethylene glycol and acrylamide) and cell adhesive (gelatin) proteins are coated on the glass substrates with a desired geometry. (b) Representative response curves obtained by averaging fluorescent intensity of 40 pixels for each cell on each frame. The small transient calcium increase due to flow perturbations has relaxed before the arrival of ATP (vertical dashed line) and only the subsequent ATP-induced calcium dynamics is analyzed. (c), (d) Typical fluorescent images of a continuous cell sheet and colonies of finite size. Scale bar 100 μm .

supplemented with 10% calf bovine serum and 1% penicillin, ATCC) before injecting into flow channels. The filled channels were incubated at 37 $^{\circ}\text{C}$ and 5% CO_2 for 24 h and then washed with growth medium to remove nonadhering cells. These steps left the cells that were growing on gelatin-coated patches to form high density (~ 1000 cells/ mm^2) colonies of desired geometry, including square patches, which we will refer to by their edge dimensions (100–300 μm), and continuous sheets covering the whole surface. After washing, we loaded calcium indicators (Fluo-4, Invitrogen) into the cells and mounted the devices on an inverted microscope (Leica SP5 confocal) for fluorescent imaging at 1 frame per sec with a 20 $\times$ oil immersion objective (numerical aperture = 0.7) [19].

ATP solutions of concentrations from 10 to 100 μM were delivered by a syringe pump at a flow rate of 60 $\mu\text{L}/\text{min}$. The flow rate was chosen to deliver ATP across the field of view within 2 sec while minimizing the flow perturbation to the cells. Cell locations were manually identified and homemade MATLAB image processing programs were used to obtain the response curves $\{R_i(t)\}$ of individual cells' average fluorescent intensity, which represent the calcium dynamics at the single cell level [Fig. 1(b)]. We note that there were small transient calcium elevations due to switching on the flow,

which relaxed quickly before the ATP arrived. We remove this section of the response curves in our analysis [6]. Figures 1(c) and 1(d) show typical calcium images of a continuous cell sheet and an array of square cell patches.

To quantify the statistical properties of the collective chemoresponse, we first define the topological distance D by Delaunay triangulation [Fig. 2(a)] such that nearest-neighbor cells have topological distances $D = 1$. This definition reflects the nature of intercellular communication and the large variations in the cell shapes [20]. For each pair of cells i and j with response curves $R_i(t)$ and $R_j(t)$, we can calculate their dimensionless cross-correlation C_{ij} :

$$C_{ij} = \frac{\int_0^T \dot{R}_i(t) \dot{R}_j(t) dt}{\sqrt{\sigma_i \sigma_j}}, \quad \text{where } \sigma_i = \int_0^T \dot{R}_i(t)^2 dt; \quad (1)$$

here, $\dot{R}_i(t) = \frac{dR_i(t)}{dt}$ is independent of the basal level fluorescence and T typically span 700 frames [21]. Also, the time average of $\dot{R}_i(t)$ can be neglected, i.e.,

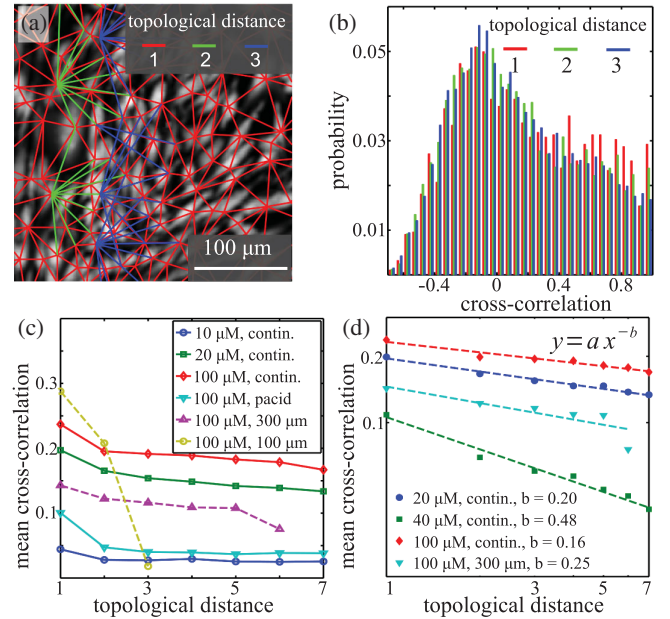


FIG. 2 (color). The mean cross-correlations vary with topological distances. (a) The triangulation based on cell locations defines the topological distance between cells. Examples of cell pairs with topological distances D ranging from 1 to 3 are linked in different colors. For clarity, only a few links of $D > 1$ are shown. (b) The normalized histogram of cross-correlations for a typical experiment (continuous sheet, $[\text{ATP}] = 100 \mu\text{M}$). 3000 cell pairs for each topological distance were used for each histogram. (c) The mean cross-correlations decay as D increases. At least 3 independent experiments were conducted for each condition (colony geometry and $[\text{ATP}]$), and ~ 3000 pairs are sampled to calculate the mean cross-correlations. (d) At moderate $[\text{ATP}]$, mean cross-correlations that decay with topological distance can be fit with power-law functions (RMSD < 0.005). Legend abbreviations: $x \mu\text{M}$, $[\text{ATP}]$; contin., continuous cell sheet; pacid, treatment with palmitic acid; $y \mu\text{m}$, edge dimensions of cell colonies with square a geometry.

$$\langle \dot{R}_i(t) \rangle_T = \frac{R_i(T) - R_i(0)}{T} \rightarrow 0, \quad (2)$$

because for most cells calcium concentrations relaxed to the initial level at the end of each experiment [Fig. 1(b)].

The cross-correlation C_{ij} measures the level of synchronization which receives positive contributions from persistent, in-phase calcium oscillations of the cell pair $\{ij\}$. For cell pairs of a given topological distance, their cross-correlations are broadly distributed with non-Gaussian characteristics [Fig. 2(b)] [22]. The shape of the distribution differs dramatically from typical excitable neural networks, where nearby cells communicate through strong-sparse and weak-dense links [23]. Although the cross-correlations exhibit large fluctuations, the distributions of C_{ij} shift to lower values at increasing topological distances Fig. 2(b)]. To quantify this observation, we define the mean cross-correlation $\bar{C}(d)$ as

$$\bar{C}(d) = \langle C_{ij} \rangle_{D(i,j)=d}, \quad (3)$$

which averages over all pairs with topological distance d . For a continuous monolayer, moderate ATP concentrations (20–100 μM) generate multiple calcium spikes for each cell and the mean cross-correlation $\bar{C}(d)$ slowly decays at larger topological distance [Fig. 2(c)]. At much lower concentrations of ATP (<10 μM), only a fraction of cells have significant calcium spikes [6], while the pair correlations near threshold ([ATP] = 10 μM) quickly decay to a small residual value, which is likely because we simultaneously stimulated all the cells in the field of view. To further confirm the role of intercellular communications, we treated the cell sheets with palmitic acid (pacid, 400 μM , 10 min) to inhibit the gap junctions [24]. In this case, pair correlations are suppressed significantly and rapidly decay to the basal level [Fig. 2(c)].

The existence of long-range correlations as indicated in Fig. 2(c) usually implies a strong dependence of system size. Indeed, although the correlations of 300 μm colonies (containing ~ 130 cells) behave similarly to a continuous monolayer, the response starts to deviate significantly when the colony sizes are reduced to 100 μm [Fig. 2(c)]. We suspect this qualitative change of the correlation behavior is linked to an underlying length scale at about 100 μm or 3–5 times the typical cell size. To better characterize the slowly decaying correlations with topological distances, we find they can be fit well with power-law functions $\bar{C}(d) = ad^{-b}$ where we have been able to vary d over about a factor of 7 [Fig. 2(d)]. To our knowledge, the long-range correlation of the individual chemical responses has not been reported before for nonexcitable mammalian cells.

Although the correlations in chemical responses between the nearby cells are heterogeneous [Fig. 2(b)], underlying spatial structures can be revealed through comparison with their reference random network. For each experiment where cell locations in a colony (either

continuous or of finite size) are known, we construct a reference Erdős-Rényi graph (ref) [25] by randomly linking qN cell pairs with topological distance $D = 1$. Here, N is the total number of nearest neighbors and $0 < q < 1$ is a cutoff fraction. The corresponding experimental graph (exp) is then constructed by selecting the strong correlators. In particular, we rank all of the nearest neighbor pairs according to their cross-correlations and link the top qN pairs. With this construction, each cell belongs to a connected cluster that contains s cells, $s \geq 1$ [26].

Now an important question can be asked: Are strong correlators randomly scattered in the cell colony, or do they tend to cluster together? To answer the question, we evaluate the probability $P(s > x)$, or simply $P(x)$, of a cell belonging to a connected cluster of size s (number of cells) bigger than x . Without loss of generality, we choose the cutoff fraction $q = 0.15$. For reference, $P_{\text{ref}}(x)$ only depends on the cell density and colony size, and exponentially decays as a function of x (Fig. 3, solid curve). The results for experimental graphs $P_{\text{exp}}(x)$ deviate systematically from P_{ref} as shown in Figs. 3(b) and 3(d). For a continuous monolayer, the strongly correlated cells tend to be in bigger clusters but are less likely to be in small fragments compared to the reference network. The crossover cluster size, at around $x \approx 4$, only weakly depends on the ATP concentrations [Figs. 3(a) and 3(b)] [27]. The same observation holds for finite size colonies, which also demonstrate a nontrivial dependence on system size: larger colonies are correlated with higher probabilities of forming big clusters [Figs. 3(c) and 3(d)]. Choosing different cutoff fractions q does not change these qualitative trends [28].

To understand the spatial organization of strongly correlated pairs, it is helpful to calculate the percolation threshold of the network: the degree k , which is the number of links connected to a cell, has its distribution determined by the intrinsic network properties and the cutoff q . If we follow a link from cell i to j , the average degree of cell j is $\kappa = \frac{\langle k^2 \rangle}{\langle k \rangle}$, the ratio of the second moment and the first moment of the degree distribution. If the cutoff q is larger than a percolation threshold q_t , we have $\kappa > 2$. In this case we can follow the link to a next cell and continue the process so that there exists a percolating cluster whose size scales with the total number of cells forming the network [29]. For the continuous monolayers stimulated by moderate concentrations of ATP (20–100 μM), we found the percolation threshold is $q_t \approx 0.1$, while their reference graphs have $q_t > 0.2$ [30]. Thus, we conclude that the self-organized networks of collective chemosensing tend to form large clusters by relaying strong correlations.

As shown in Fig. 2(b), the distribution of pair cross-correlations are far from Gaussian. In fact, the mean $\bar{C} = \text{avg}[C_{ij}]_{D(i,j)=1}$ and variance $\text{var}[C_{ij}]_{D(i,j)=1}$ of nearest neighbors' cross-correlations all fall on the same line as

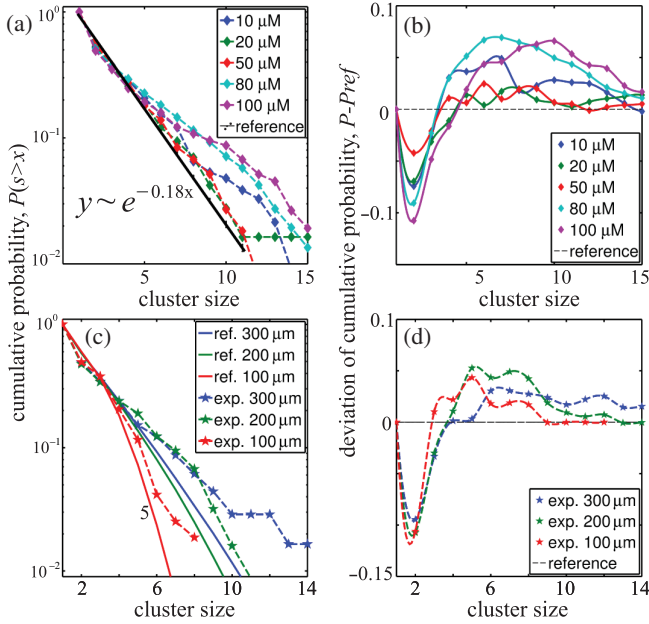


FIG. 3 (color). The cluster distribution of strong correlators for cutoff fraction $q = 0.15$. (a) The probability $P(s > x)$ of a cell belonging to a cluster larger than size x , where x is the number of cells in a cluster formed by linking strong correlators. At least 3 experiments at each given [ATP] were used to obtain the average probabilities. For each experiment, 2000 realizations of its corresponding reference network were simulated and averaged to obtain the reference probability distributions P_{ref} . For continuous monolayers, and given cutoff fraction, P_{ref} only depends weakly on [ATP]. (b) The deviation of probability distributions between experimental networks and their corresponding reference graphs $P_{\text{exp}} - P_{\text{ref}}$ for continuous cell sheets at different [ATP]. (c), (d) The probability distributions and the difference between experiment and their corresponding reference networks for finite-sized colonies stimulated with $100 \mu\text{M}$ ATP. Legend abbreviations: as in Fig. 2; exp., experimental results; ref., simulation results of reference networks.

shown in Fig. 4 (filled symbols). This result means that the ratio $\frac{\text{var}[C_{ij}]}{\text{avg}[C_{ij}]}$ is a fundamental quantity independent of ATP concentrations and colony sizes. The fact that the variance is proportional to the mean usually implies an underlying Poisson process [31]. Therefore, we hypothesize that the number of gap junctions formed between nearby cells follows a Poisson distribution. With this assumption, the elementary contribution $c_0 \approx 0.3$ of each gap junction to the cross-correlation is determined by the slope of the line in Fig. 4 [32].

For simplicity, we consider only IP3 communications through a single gap junction, which weakly affect the paces of each cell's transient calcium oscillations. We expect the elementary correlation c_0 is then a function of the cell volume V , the unitary conductance k of a single gap junction (number of IP3 molecules transported per unit time and unit concentration difference), and the typical

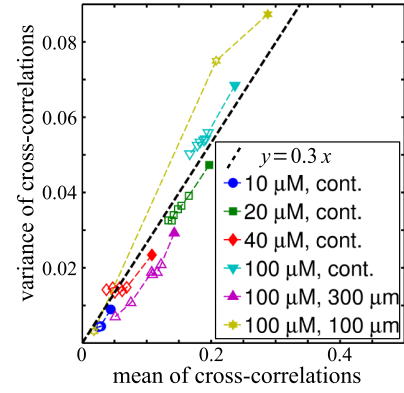


FIG. 4 (color online). The statistics of cross-correlations. Means and variances of nearest-neighbor cross-correlations (filled symbols) for different ATP concentrations and colony sizes fall near a line $y = 0.3x$. Cross-correlations of larger topological distances (empty symbols) scatter around the same line. Each point is calculated from ~ 3000 cell pairs from at least 3 experiments of each given condition. Legend abbreviations are the same as in Fig. 2.

frequency ω_0 of the calcium oscillation. Using only these physical ideas, from dimensional analysis we expect

$$c_0 = \alpha \left(\frac{k}{V\omega_0} \right)^\beta. \quad (4)$$

Here, α is a numerical prefactor of order 1, $V \sim 2 \times 10^{-7} \text{ mm}^3$, $\omega_0 \sim 0.05 \text{ Hz}$ [6], and $\beta \approx 1$, as we have confirmed by extending a calcium-IP3 model [33] with gap junction communications [34]. Using Eq. (4), we estimate the unitary conductance of each gap junction to be $k \sim 3 \times 10^{-9} \text{ mm}^3/\text{s}$, which agrees well with direct experimental measurements [35].

The variances and means of larger topological distances also fall on the same line [$1 < D(i, j) < 7$; Fig. 4, empty symbols]. We hypothesize that effective communication is established between non-neighboring cells due to the long-range correlations. Thus, smaller $\bar{C}(d)$ at larger d can be understood as a decrease in the number of effective communicating channels at larger d .

In this Letter, we have discussed the network characteristics of collective chemosensing by fibroblast cells exposed to external ATP stimuli. By combining micro-patterning and microfluidics, we investigated the effects of both stimuli concentrations and cell colony sizes. We find the cross-correlation function between the responses of individual cells decayed with topological distance as power law for large colonies and much faster for smaller colonies. The exponentials of the power-law decay [Fig. 2(d)] fall in a similar range as those reported for bacteria suspension [36] and flocking birds [37,38]. By comparing with random networks, we found strongly correlated cell pairs tend to form clusters and statistically are more likely to exceed the percolation threshold, which could lead to clusters that scale with the size of the system. At a given topological

distance, the cross-correlations exhibit characteristics of Poisson distributions, where the ratio of the mean and variance are independent of ATP concentration and cell colony size. This result allows us to estimate the conductance of a single gap junction, which is in good agreement with direct experimental measurements. Our study highlights the rich spatiotemporal structures that are built into the self-organized networks of nonexcitable mammalian cells. These results help to understand the information encoding and signal transduction in multicellular organisms, which regulate a broad range of physiological functions such as tissue remodeling and immunological responses.

We are grateful to Dr. Maxime Deforet and Professor Pascal Silberzan for sharing micropatterning techniques. The project described was supported by Firmenich SA, Corporate R&D, Geneva, Switzerland, through a research grant.

*Present address: Department of Physics, Oregon State University, Corvallis, Oregon 97331, USA.

- [1] H. Kitano, *Science* **295**, 1662 (2002).
- [2] D. Vaudry, P.J.S. Stork, P. Lazarovici, and L.E. Eiden, *Science* **296**, 1648 (2002).
- [3] H. Shibuya, M. Yoneyama, J. Ninomiya-Tsuji, K. Matsumoto, and T. Taniguchi, *Cell* **70**, 57 (1992).
- [4] M. Freeman, *Nature (London)* **408**, 313 (2000).
- [5] M.M. Mueller and N.E. Fusenig, *Nat. Rev. Cancer* **4**, 839 (2004).
- [6] B. Sun, J. Lembong, V. Normand, M. Rogers, and H.A. Stone, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 7759 (2012).
- [7] T. Gregor, K. Fujimoto, N. Masaki, and S. Sawai, *Science* **328**, 1021 (2010).
- [8] S. Sawai, P.A. Thomason, and E.C. Cox, *Nature (London)* **433**, 323 (2005).
- [9] B.N. Vasiev, P. Hogeweg, and A.V. Panfilov, *Phys. Rev. Lett.* **73**, 3173 (1994).
- [10] C. Léon, B. Hechler, M. Freund, A. Eckly, C. Vial, P. Ohlmann, A. Dierich, M. LeMeur, J. Cazenave, and C. Gachet, *J. Clin. Invest.* **104**, 1731 (1999).
- [11] S. Yitzhaki, A. Shainberg, Y. Cheporko, B.A. Vidne, A. Sagie, K.A. Jacobson, and E. Hochhauser, *Biochem. Pharmacol. (Amsterdam, Neth.)* **72**, 949 (2006).
- [12] M.J. Berridge and R.F. Irvine, *Nature (London)* **341**, 197 (1989).
- [13] W.H. Evans and P.E.M. Martin, *Mol. Membr. Biol.* **19**, 121 (2002).
- [14] G. Burnstock and G.E. Knight, *Int. Rev. Cytol.* **240**, 301 (2004).
- [15] S. Orrenius, B. Zhivotovsky, and P. Nicotera, *Nat. Rev. Mol. Cell Biol.* **4**, 557 (2003).
- [16] A.L. Monaco, S. Gulinelli, G. Castellino, A. Solini, D. Ferrari, R.L. Corte, F. Trotta, and F.D. Virgilio, *Ann. Rheum. Dis.* **66**, 1124 (2007).
- [17] A. Tourovskaia, X. Figueroa-Masot, and A. Folch, *Nat. Protoc.* **1**, 1092 (2006).
- [18] M. Deforet and P. Silberzan (to be published).
- [19] See Supplemental Material at <http://link.aps.org/supplemental/10.1103/PhysRevLett.110.158103> for movies of typical experiments.
- [20] See Supplemental Material at <http://link.aps.org/supplemental/10.1103/PhysRevLett.110.158103> (Secs S1, S2) for more discussions on Delauney triangulation.
- [21] See Supplemental Material at <http://link.aps.org/supplemental/10.1103/PhysRevLett.110.158103> (Sec. S3) for analysis on the effect of time discretization.
- [22] See Supplemental Material at <http://link.aps.org/supplemental/10.1103/PhysRevLett.110.158103> (Sec. S4) for more statistical characteristics.
- [23] J. Teramae, Y. Tsubo, and T. Fukai, *Sci. Rep.* **2**, 485 (2012).
- [24] E. Lavado, L.I. Sánchez-Abarca, A. Tabernero, J.P. Bolaños, and J.M. Medina, *J. Neurochem.* **69**, 721 (1997).
- [25] A. Barrat, M. Barthélemy, and A. Vespignani, *Dynamical Processes on Complex Networks* (Cambridge University Press, Cambridge, England, 2008).
- [26] See Supplemental Material at <http://link.aps.org/supplemental/10.1103/PhysRevLett.110.158103> (Sec. S5) for examples of network construction.
- [27] See Supplemental Material at <http://link.aps.org/supplemental/10.1103/PhysRevLett.110.158103> (Sec. S6) for more details.
- [28] See Supplemental Material at <http://link.aps.org/supplemental/10.1103/PhysRevLett.110.158103> (Sec. S7) for the probability distribution at different cutoffs q .
- [29] R. Cohen and S. Havlin, *Complex Networks* (Cambridge University Press, Cambridge, England, 2010).
- [30] See Supplemental Material at <http://link.aps.org/supplemental/10.1103/PhysRevLett.110.158103> (Sec. S8) for the percolation analysis of typical experiments.
- [31] N.L. Johnson, A.W. Kemp, and S. Kotz, *Univariate Discrete Distributions* (Wiley-Interscience, Hoboken, NJ, 2005), 3rd ed.
- [32] V. Bhandawat, J. Reiser, and K. Yau, *Science* **308**, 1931 (2005).
- [33] Y. Tang and H.G. Othmer, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7869 (1995).
- [34] See Supplemental Material at <http://link.aps.org/supplemental/10.1103/PhysRevLett.110.158103> (Sec. S9) for the details of the numerical model.
- [35] J.M. Nitsche, H. Chang, P.A. Weber, and B.J. Nicholson, *Biophys. J.* **86**, 2058 (2004).
- [36] X. Chen, X. Dong, A. Béer, H.L. Swinney, and H.P. Zhang, *Phys. Rev. Lett.* **108**, 148101 (2012).
- [37] A. Cavagna, A. Cimarelli, I. Giardina, G. Parisi, R. Santagati, F. Stefanini, and M. Viale, *Proc. Natl. Acad. Sci. U.S.A.* **107**, 11 865 (2010).
- [38] W. Bialek, A. Cavagna, I. Giardina, T. Mora, E. Silvestri, M. Viale, and A.M. Walczak, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 4786 (2012).