Inoculum-density dependent growth reveals inherent cooperative effects and stochasticity in cancer cell cultures

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We document the initial-density dependence of the growth rate achieved by Jurkat cell cultures in a standard growth medium with fixed carrying capacity. As the density $N_0$ of the inoculum varies over 4 orders of magnitude, three distinct growth regimes appear. At small $N_0$, the growth rate $\lambda$ is roughly constant and displays small sample-to-sample variability. Upon increasing $N_0$, $\lambda$ increases slowly together with fluctuations. Finally, after peaking at an intermediate $N_0$, it gets smaller and more homogeneous across cultures as the inoculum density converges to the carrying capacity of the medium. A minimal, deterministic, population-based mathematical model explains the observed qualitative features assuming (in agreement with data) the presence of a weak positive correlation between cell density and proliferation rate. These results support the idea that cell cultures can preserve a memory of the initial condition, possibly via contact interactions or through metabolic coupling.
I. INTRODUCTION

The inoculum density has long been known to affect different features of cell growth in a variety of cell types and organisms. Following Rubin’s pioneering work on chick embryo cells [1], initial density-dependent traits have been unveiled, among others, in bacterial [2–5], insect [6], plant [7–9] and mammalian [10–12] cell cultures. Across different studies, such traits were found to include both physiological characteristics –like the doubling time, the maximum attainable cell concentration, or the duration of the lag phase– and metabolic signatures –such as the capacity to produce specific compounds. The growth rate, in particular, can appear to increase [6, 9] or decrease [8, 12] (see also [13]) as the density of the inoculum changes. In cases where it remains roughly constant, its fluctuations can undergo significant shifts in magnitude upon modulating the initial seed [4, 7]. While it is a priori hard to compare growth properties across such a broad spectrum of cell types and experimental protocols, the very fact that these regularities are observed in a variety of different systems and conditions suggests that, at least in part, they could stem from a common set of mechanisms that are inherent in the culture’s initial condition and whose effects are preserved during the subsequent history of the population.

Many background causes can lead to memory in cell growth. For instance, the standard logistic growth model à la Baranyi [14] suggests that the growth medium can impose a well-defined initial-density dependence on the growth rate through a finite carrying capacity $K$. In particular, the growth rate should be expected to decline as the initial seed approaches $K$, as quantified e.g. in [15]. More generally, initial density dependence can be understood in terms similar to those employed for generic density-dependent features described by the Allee effect [16, 17]. In this light, a decrease of growth rate with the initial cell density can occur whenever cells are subject to competition with density-dependent strength due e.g. to medium conditioning or finite resources. On the other hand, a positive correlation between the growth rate and the initial density can point to the existence of cooperative, density-dependent effects. Interestingly, memory and history-dependence have recently been investigated as factors influencing exit from quiescence in model REF/E23 cells [18] and competence in bacteria [19]. In the latter case, quorum sensing was identified as a central coordinating mechanism.

In this work, we have quantified the initial-density dependence of the growth rate and of
its fluctuations in a widely used cancer cell type (Jurkat cells), varying the inoculum size over four orders of magnitude. We uncover a complex dependence of the effective growth rate of populations of identically prepared cultures on their initial density, with an initial slow increase followed by a rapid decrease as the initial density gets closer to the carrying capacity of the growth medium. In particular, the mean growth rate computed over multiple biological replicates appears to peak in a specific range of values for the inoculum density. In addition, a considerable increase in growth-rate variability is observed upon increasing the initial density of the population starting from small values. Most notably, the size of fluctuations is found to be maximal when the mean growth rate peaks. Our findings are explained by a minimal mathematical model of population growth that accounts for just two empirically validated ingredients, namely (a) weak cooperative inter-cellular effects with strength that can fluctuate stochastically across different cultures, and (b) a finite carrying capacity.

II. RESULTS

A. Systematic cell counting reveals unimodal growth rate distribution for identically prepared cells and constant carrying capacity

To assay for growing populations of cells we developed a MATLAB-based code to analyse multiple snapshots (images) of the growing population, and count cells over time. The pipeline uses built-in MATLAB functions to perform image segmentation and distinguish dead cells from viable ones. Plotting the logarithm of the number of viable cells versus time and fitting the data with a standard logistic function (see Materials and Methods), we could identify the different phases of growth, in particular the exponential (log) and saturation phases.

As a first outcome of this analysis, we quantified the mean growth rate of the population in the log phase for every growth curve obtained from 145 different experiments. Figure 1(a) shows that the distribution of growth rates thus obtained is a well-defined unimodal distribution, peaked around $\lambda \simeq 0.025 \text{ h}^{-1}$.

As our experiments were started from a broad class of distinct initial conditions (defined by the value $N_0$ of the inoculum density), we checked consistency of the values of the carrying
capacity over our entire dataset. Figure 1(c) shows that the parameter $A$ that defines the saturation level in the logistic fits we employed (see Methods) decreases linearly with $\log(N_0)$. As the logarithm of the carrying capacity $K$ is given by $A + \log(N_0)$, this confirms that $K$ is indeed constant and independent on the seeding condition across different experiments.

B. The mean growth rate and the growth rate fluctuations are modulated by the initial cell density

When sorted with respect to the initial density $N_0$, growth rates $\lambda$ display a non-trivial behaviour. Figure 1(b) shows that, upon increasing $N_0$, the mean growth stays roughly constant for small $N_0$, then increases and peaks for intermediate values of $N_0$, and finally decreases linearly as $N_0$ gets closer to the carrying capacity $K$. Moreover, the standard deviation $\sigma_\lambda$ shows a maximum approximately for the same intermediate values of $N_0$ where the mean growth rate is maximized, see Figure 1(d). Expectedly, as $N_0$ approaches the maximal cell density sustainable per fixed volume of nutrient, the growth rate decreases.

We checked if the growth rate dependency on $N_0$ were somehow related to the previous history of the culture, measured through the lag time $t_{\text{lag}}$. However, there is no immediate dependence of $\lambda$ on the lag time, see Figure 1(e). This suggests that $N_0$ is the only variable that resolves the growth rates.

The overall behaviour of the mean growth rate as a function of $N_0$ is not consistent with an augmented limitation in the amount of resources per increased initial population size. In particular, the fact that the mean $\lambda$ is enhanced by an increase of $N_0$ suggests that, when $N_0$ gets sufficiently large, the population can globally benefit from inter-cellular cooperation, consistently with a weak Allee effect scenario.

C. A minimal mathematical model explains the empirical picture

Experimental findings can be partially understood in terms of a minimal, deterministic mathematical model of population growth. Our central assumption is that cells evolve in an environment characterized by a fixed, $N_0$-independent carrying capacity $K$ (consistently with Figure 1(c) and that the population growth rate correlates positively with the cell density at small densities, as described by the weak Allee effect [17]. Generalizing the standard model
of the Allee effect discussed e.g. in [20], the population size (or density) \( N \) can be thought to evolve according to
\[
\frac{dN}{dt} = rN \left( 1 - \frac{N}{K} \right) \left( \frac{N}{K} \right)^a,
\]
where \( r \) denotes an intrinsic time scale, \( K \) the carrying capacity of the growth medium and \( a \geq 0 \) an exponent describing the strength of density dependence. The case \( a = 0 \) corresponds to standard logistic growth in the presence of a finite carrying capacity [14]. For \( a > 0 \), the population growth rate \( \frac{dN}{dt} \) is low for small enough the population size \( N \) and increases significantly when \( N \) exceeds a (soft) threshold level, effectively accounting for stronger and mutually beneficial inter-cellular cooperation. This effect sets in at higher densities the larger the value of \( a \) (see Figure 2(a), inset). On the other hand, when \( N \) gets sufficiently large growth slows down as the carrying capacity is approached.

Population size-growth rate maps obtained from experimental growth curves are consistent with values of \( a \) close to 1 (see Figure 2(a)), suggesting that indeed cells grow in a weakly cooperative manner. However, to account for stochastic effects in environmental, demographic and/or intracellular factors, we assume that \( a \) can fluctuate across experiments. In other terms, different colonies initiated at the same inoculum density \( N_0 \) are assumed to evolve with slightly different values of \( a \). In the simplest case, we have considered a uniform distribution for \( a \) defined by a fixed mean \( \bar{a} = 1 \) and by relative fluctuations \( \sigma_a/\bar{a} = 0.3 \), independently of \( N_0 \). (The specific value of \( \sigma_a/\bar{a} \) does not affect the qualitative outlook of results.)

Trajectories of (1) can be easily generated numerically for any given initial density \( N_0 \) and for a given (randomly generated) \( a \) and, provided \( N_0 \) is sufficiently far from the carrying capacity \( K \), one can measure the growth rate defined as
\[
\lambda = \frac{\log(N_{\log}) - \log(N_{\text{lag}})}{t_{\log} - t_{\text{lag}}},
\]
where the duration of the lag phase \( (t_{\text{lag}}) \) and the exit time from the log phase \( (t_{\log}) \) are defined as in Figure 3 while \( N_{\text{lag}} = N(t_{\text{lag}}) \) and \( N_{\log} = N(t_{\log}) \). Figure 2(b) shows that the mean growth rate obtained, for each \( N_0 \), by averaging over 200 independent choices of \( a \) displays a slow increase with \( N_0 \) before peaking and then rapidly decaying as \( N_0 \) gets closer to \( K \). (Note that upon changing the value of \( K \) the mean growth rate simply re-scales to adjust to the allowed range of values of \( N_0 \). Therefore results are shown for a representative choice of \( K \).) At the same time (see Figure 2(c)), the standard deviation of \( \lambda \) behaves in
a similar way, increasing with $N_0$ at small densities, peaking and then rapidly decreasing as $K$ is approached. One sees that, in spite of its crudeness, this model can reproduce the qualitative features of the growth rate and its fluctuations observed in experiments. The major discrepancy is found in the behaviour of fluctuations at small densities. The empirical scenario is however fully captured by assuming that relative fluctuations of $a$ correlate with $N_0$. In specific, empirical evidence supports a picture in which the fluctuations of $a$ increase with $N_0$ (see Figure 2(c) for a realization of this scenario). While it is hard to pinpoint the specific factors that might influence the value of the parameter $a$ in different samples, this suggests that the soft density threshold for proliferation might be affected by contact interactions or medium conditioning, both of which may be expected to be more significant and subject to stronger variability in larger inocula. Overall, our minimal model therefore shows that empirical data are consistent with certain specific features of population growth (like the exponent $a$) being themselves affected by the initial conditions.

III. DISCUSSION

In recent years, a variety of technological and computational advances have allowed for considerable progress in the quantification of inter-cellular variability within individual populations [21–23]. Such studies have accessed a previously unexplored level of biological complexity by directly monitoring the behaviour of single cells and opened the way to the resolution of some long-standing cell-biology puzzles, such as the origin of cell size homeostasis [24]. In spite of these findings, it is not unreasonable to expect different populations grown in identical media to display very similar growth patterns. The available information on sample-to-sample fluctuations in populations of cells suggests however that this is not necessarily the case. In particular, several studies have singled out the inoculum density as a factor in determining the overall growth behaviour of colonies, identifying, across a broad range of systems, regimes in which the initial population size correlates with the population growth rate, either positively or negatively.

This study has presented a large-scale picture of how growth properties, specifically the growth rate and its fluctuations, are modulated by the inoculum density in a widely used cancer cell line. We performed 145 experiments in controlled media, the only difference consisting in the fact that we initialized cultures from different inocula, covering roughly
four orders of magnitude in size. Starting from low initial densities \( N_0 \), the mean growth rate slowly increases with \( N_0 \), displays a maximum and then (expectedly) decreases as the initial population gets closer and closer to the carrying capacity of the medium. Growth rate fluctuations show a similar pattern, with a marked increase followed by a rapid decrease, and the maximum is attained at values of \( N_0 \) for which the mean growth rate peaks.

These results strongly indicate that the growth rate achieved for small-to-moderate initial densities can be influenced by the establishment of cooperative interactions within the populations. On the other hand, the finite carrying capacity suffices to explain the behaviour for larger values of \( N_0 \). Indeed, following traditional modeling frameworks employed to describe cell populations, we developed a simple deterministic mathematical model that can fully recapitulate the observed behaviour.

The complex role of initial conditions highlighted here implicates memory and (possibly) history-dependence as potential determinants for the growth behaviour of mammalian cell cultures. Perhaps unsurprisingly, a precise quantification of their role in controlled experiments is thus far lacking. Yet, they might bear significant consequences on different levels. It would be particularly important to enrich the set of experimental evidence by cross-correlating the inoculum density to the characteristics of the log-phase and to those of the preceding adaptation period and by analyzing (or re-analyzing) these issues in different cell types. Moreover, it would be important to identify the population-level mechanisms underlying these results more clearly. Indeed, if the qualitative patterns uncovered in the present work are generic (i.e. not specific to the cell type we investigated), the same mechanisms are likely to be active in different contexts. In this light, a more thorough (and perhaps parameter-rich) modeling approach, would go beyond the minimal framework used here in shedding light on these findings.

IV. MATERIALS AND METHODS

A. Cell culture and counting

Jurkat cells (clone E6-1, ATCC) were cultured in RPMI-1640 Medium (Gibco) supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C and in 5%CO₂. Cells growing exponentially were then seeded at different concentrations in 6-well plates.
(Falcon) in a fixed volume of the previous medium and counted every 24 hours until the saturation was reached. The counting procedure applies as follows: cells were pipetted gently and 30µl of cells and medium were mixed with 30µl of methylene blue (1% in PBS). Blue cells correspond to death cells while bright round-shaped cells correspond to alive ones. The mixture is then transferred in three single-use Burker’s chambers. For each chamber five brightfield photos are taken (15 different photos for each experiment at a given time) with an Axiovert Zeiss inverted microscope (objective 10x). The pictured surfaces correspond to a volume of 10^{-4} ml. The photos are then analyzed via a MATLAB-based image analysis code that implements built-in function to count alive cells.

B. Data fitting procedure

Plotting the logarithm of the number $N$ of alive cells per ml normalized by the initial concentration $N_0$ versus time, we can recover the growth curve of the population. In order to quantitatively determine the different phases of growth, namely the adaptation, exponential and saturation, we fit the growth curve with the logistic function

$$
\log \frac{N}{N_0} = \frac{A}{1 + \exp \left[ \frac{4\mu_{\text{max}}}{A}(t_{\text{lag}} - t) + 2 \right]}. 
$$

(3)

The parameters $A$, $t_{\text{lag}}$ and $\mu_{\text{max}}$ are respectively the saturation, the lag time and the maximum growth rate. The maximum growth rate corresponds to the slope of the tangent to the inflection point. The lag time corresponds to the end of the adaptation phase, that is the beginning of the exponential one. In order to determine the end of the exponential phase we consider the time corresponding to the intersection between the saturation $A$ and the tangent to the inflection point, see Figure 3. We then fit the data within this range with a straight line whose slope corresponds to the effective growth rate $\lambda$ of the population in the exponential phase.

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[1] Rein, A., & Rubin, H. (1968). Effects of local cell concentrations upon the growth of chick embryo cells in tissue culture. Experimental Cell Research, 49(3), 666-678.

[2] Postma, J., Hok-A-Hin, C. H., & Voshaar, J. O. (1990). Influence of the inoculum density on the growth and survival of Rhizobium leguminosarum biovar trifolii introduced into sterile and non-sterile loamy sand and silt loam. FEMS Microbiology Letters, 73(1), 49-57.

[3] Coleman, M. E., Tamplin, M. L., Phillips, J. G., & Marmer, B. S. (2003). Influence of agitation, inoculum density, pH, and strain on the growth parameters of Escherichia coli O157: H7–relevance to risk assessment. International Journal of Food Microbiology, 83(2), 147-160.

[4] Irwin, P. L., Nguyen, L. H. T., Paoli, G. C., & Chen, C. Y. (2010). Evidence for a bimodal distribution of Escherichia coli doubling times below a threshold initial cell concentration. BMC Microbiology, 10(1), 207.

[5] Koutsoumanis, K. P., & Lianou, A. (2013). Stochasticity in colonial growth dynamics of individual bacterial cells. Applied and Environmental Microbiology, 79(7), 2294-2301.

[6] Marteijn, R. C. L., Oude-Elferink, M. M. A., Martens, D. E., De Gooijer, C. D., & Tramper, J. (2000). Effect of Low Inoculation Density in the Scale-Up of Insect Cell Cultures. Biotechnology Progress, 16(5), 795-799.

[7] Van Gulik, W. M., Nuutila, A. M., Vinke, K. L., Ten Hoopen, H. J., & Heijnen, J. J. (1994). Effects of carbon dioxide, air flow rate, and inoculation density on the batch growth of Catharanthus roseus cell suspensions in stirred fermentors. Biotechnology Progress, 10(3), 335-339.

[8] Kanokwaree, K., & Doran, P. M. (1997). Effect of inoculum size on growth of Atropa belladonna hairy roots in shake flasks. Journal of Fermentation and Bioengineering, 84(4), 378-381.

[9] Carvalho, E. B., & Curtis, W. R. (1999). The effect of inoculum size on the growth of cell and root cultures of Hyoscyamus muticus: implications for reactor inoculation. Biotechnology and Bioprocess Engineering, 4(4), 287-293.

[10] Ozturk, S. S., & Palsson, B. Ø. (1990). Effect of initial cell density on hybridoma growth, metabolism, and monoclonal antibody production. Journal of Biotechnology, 16(3-4), 259-278.
[11] Rodriguez, E. N., Perez, M., Casanova, P., & Martinez, L. (2001). Effect of Seed Cell Density on Specific Growth Rate Using CHO Cells as Model. In: Animal Cell Technology: From Target to Market (pp. 434-437). Springer Netherlands.

[12] Gregorio, A. C., Fonseca, N. A., Moura, V., Lacerda, M., Figueiredo, P., Simoes, S., Dias, S., & Moreira, J. N. (2016). Inoculated Cell Density as a Determinant Factor of the Growth Dynamics and Metastatic Efficiency of a Breast Cancer Murine Model. PloS ONE, 11(11), e0165817.

[13] dtp.cancer.gov/discovery_development/nci-60/cell_list.htm

[14] Baranyi, J., & Roberts, T. A. (1994). A dynamic approach to predicting bacterial growth in food. International Journal of Food Microbiology, 23(3-4), 277-294.

[15] De Martino, D., Capuani, F., & De Martino, A. (2017). Quantifying the entropic cost of cellular growth control. Physical Review E 96, 010401(R).

[16] Hixon, M. A., & Johnson, D. W. (2009). Density dependence and independence. In: Encyclopedia of Life Sciences (eLS). John Wiley & Sons Ltd, Chichester (UK). [http://www.els.net [doi: 10.1002/9780470015902.a0021219]

[17] Stephens, P. A., Sutherland, W. J., & Freckleton, R. P. (1999). What is the Allee effect? Oikos, 185-190.

[18] Wang, X., Fujimaki, K., Mitchell, G. C., Kwon, J. S., Della Croce, K., Langsdorf, C., Zhang, H. & Yao, G. (2017). Exit from quiescence displays a memory of cell growth and division. Nature Communications, 8, 321.

[19] Moreno-Gomez, S., Sorg, R. A., Domenech, A., Kjos, M., Weissing, F. J., van Doorn, G. S., & Veening, J. W. (2017). Quorum sensing integrates environmental cues, cell density and cell history to control bacterial competence. Nature Communications, 8, 854.

[20] Boukal, D. S., & Berec, L. (2002). Single-species models of the Allee effect: extinction boundaries, sex ratios and mate encounters. Journal of Theoretical Biology, 218(3), 375-394.

[21] Wang, P., Robert, L., Pelletier, J., Dang, W. L., Taddei, F., Wright, A., & Jun, S. (2010). Robust growth of Escherichia coli. Current Biology, 20(12), 1099-1103.

[22] Kiviet, D. J., Nghe, P., Walker, N., Boulineau, S., Sunderlikova, V., & Tans, S. J. (2014). Stochasticity of metabolism and growth at the single-cell level. Nature, 514(7522), 376-379.

[23] Wallden, M., Fange, D., Lundius, E. G., Baltekin, Ö., & Elf, J. (2016). The synchronization of replication and division cycles in individual E. coli cells. Cell, 166(3), 729-739.
[24] Si, F., Li, D., Cox, S. E., Sauls, J. T., Azizi, O., Sou, C., Schwartz, A. B., Erickstad, M. J., Jun, Y., Li, X. & Jun, S. (2017). Invariance of initiation mass and predictability of cell size in Escherichia coli. Current Biology, 27(9), 1278-1287.

[25] Zwietering, M. H., Jongenburger, I., Rombouts, F. M. & Van’t Riet, K. (1990). Modeling of the bacterial growth curve. Applied and Environmental Microbiology, 56(6), 1875-1881.
FIG. 1: Dependence of growth features on the initial population density. (a) Distribution of effective growth rates obtained in 145 distinct experiments. (b) Effective growth rates $\lambda$ versus the initial density of the population $N_0$. Red markers denote the values of $\lambda$ obtained from each of the 145 growth curves. The error bar quantifies the error due to the fitting procedure divided by the square root of the number of fitted data. Black markers represent the mean values of $\lambda$ obtained by binning over $N_0$, with amplitude given by the black bars. (c) Saturation parameter $A$ obtained from the growth curves versus the initial density of the population $N_0$. Error bars correspond to the error of the fit. The observed trend is compatible with a straight line with slope $-1$ (black line). The carrying capacity $K$ is given by $\log(K) = A(N_0) + \log(N_0)$ and turns out to be constant and equal to the value marked by the black dashed line, namely $K \simeq 7 \times 10^6$ cells per ml. (d) Standard deviation of the growth rate, $\sigma_\lambda$, versus the initial density $N_0$. The black bars represent the amplitude of the binning employed over $N_0$. (e) Effective growth rates $\lambda$ versus the initial density of the population $N_0$ coloured with respect to the measured lag time $t_{lag}$. 
FIG. 2: Model results. (a) Population growth rate \( \frac{dN}{dt} \) versus population size \( N \) map obtained from experimental growth curves \( N \) versus \( t \). Only data for small \( N \) (far from the carrying capacity) are shown. Inset: Population growth rate \( \frac{dN}{dt} \) versus population size \( N \) map obtained from the model described by Eq. (1) for different values of \( a \) \((a = 0, 1, 2, 5) \) increasing in the direction of the arrow). The carrying capacity was set to the representative value \( K = 1000 \). (b) Mean growth rate versus inoculum density obtained from Eq. (1) with \( K = 5 \times 10^5 \) by averaging over 200 independent choices of \( a \), assuming a uniform distribution with \( \bar{a} = 1 \) and constant \((N_0\)-independent\) variance \( \sigma_a = 0.3 \) (green curve) or variance increasing with \( N_0 \) as a Hill function, \( \sigma_a = \sigma_{\text{min}} + (\sigma_{\text{max}} - \sigma_{\text{min}}) \frac{N_0^h}{N_0^h + C^h} \), with representative parameters \( \sigma_{\text{min}} = 0 \), \( \sigma_{\text{max}} = 0.3 \), \( h = 3/2 \) and \( C = 30000 \) (blue curve). (c) Standard deviation of the growth rate versus inoculum density for the data of panel (b).

FIG. 3: Data fitting and growth phases. Representative growth curve (blue circles) shown with the corresponding logistic fit (blue curve), with the lag time \( t_{\text{lag}} \), the maximum growth rate \( \mu_{\text{max}} \) and the saturation parameter \( A \) emphasized explicitly. The data error bar is given by propagating the error over \( \log \frac{N}{N_0} \) and assuming \( \sqrt{N} \) to be the error of the number of cells \( N \). The time \( t_{\text{log}} \) corresponds to the end of the log phase.