The effectiveness of glass beads for plating cell cultures

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Abstract

Cell plating and subsequent colony growth is an important laboratory procedure in microbiology. A common protocol for cell culture plating in Petri dishes uses glass beads to spread cells on the surface of the solid growth media. Here we perform an experimental analysis and mathematical modelling of cell plating. We introduce a new class of billiard - shifting billiard, which serves as a conceptual abstraction of the cell plating with glass beads. Numerical analysis of the dynamics of this simple yet efficient billiard, indicates a close relationship between plate movement and quality of colony distribution. Yeast *Saccharomyces cerevisiae* suspensions at different cell concentrations were used to confirm our theoretical observations.

1 Introduction

Microbiology contributes to a wide range of practical applications: medicine, pharmaceuticals, biological industry, agriculture, veterinary science, biotechnology and food science all rely substantially on microbiology [1]. Cell plating and subsequent colony growth is an important laboratory procedure in microbiology. The goal of cell plating is to separate cells contained within a small sample volume by homogeneously distributing the cell suspension over the surface of a plate. This results in the formation of discrete colonies after incubation that can subsequently be enumerated or subjected to further analysis. Counting the number of colonies is used in many cell culture protocols: constructing gene-knockout libraries [2]; cell-based DNA cloning [3]; measuring progenitor stem cell content [4]; testing resistance to drugs [5]; detection of bacteria in food samples or clinical specimens [6]. Successful cultivation of cells depends

critically on the choice of appropriate growth media, cell plating method and incubation conditions.

A colony is defined as a visible cluster of cells growing on the surface of a medium, presumably derived from a single cell. These single progenitor cells are also called Colony Forming Units (CFU), which provide an estimate of the number of viable cells. The more homogeneously cells are spread on the surface during plating, the better separated the resulting cell colonies, leading to a more precise CFU count. It is expected that the number of colonies formed is linearly proportional to the concentration of viable cells in the suspension, provided that the suspension was mixed and spread well. If the suspension is not spread well, clusters of cells will be formed which visually resemble a single colony and therefore will be enumerated as a single colony. To proceed with these they need to be restreaked to get single colonies which takes extra incubation time.

There are two strategies for cell plating: spread-plating with a turntable rod or spread-plating with glass beads [7]. Using the first method, a small volume of a cell suspension is spread over the plate surface using a sterile bent glass rod as the spreading device. The second method, which is the subject of our study, involves shaking glass beads over the surface of the plate. This technique is also known as the Copacabana method [8, 9].

The protocol for using sterile glass beads for dispersion of cells on solid media has the following steps [7, 8]: (1) a cell suspension is dispensed onto the middle of a round Petri dish containing solid media; (2) spherical glass beads are poured into the middle of the plate; (3) the plate lid is closed; (4) the plate is agitated with a shaking motion so that the glass beads roll over the entire surface of the plate; (5) the plate is then inverted to remove the beads before incubation.

In this study we are interested in how a plate should be agitated in order to achieve optimal spread of CFUs. For example, it is recommended that a swirling motion should be avoided to prevent the cells ending up at the plate edge [7]. Here we apply a model from statistical mechanics - billiard - to investigate this problem.

Billiards are mathematical models describing a dynamical system where one or more particles moves in a container and collides with its walls [10]. Particle movement is defined by the following elastic collision rule: the angle of reflection is equal to the angle of incidence [11]. The dynamics of the particle closely relates to the shape of the billiard boundary and may vary from regular to chaotic [10]. Recently various classes of billiard with perturbed boundaries [12] or twisted walls have been introduced [13]. Analysis showed that behavior of such dynamical systems was sensitive even to a small perturbation of the boundary shape. We are interested in the dynamics of point particles moving in a circular billiard with shifting boundaries. We numerically investigate the behavior of the system and characterize the output in terms of a box count. Our analysis is based on a simple case and the practical implementation confirms our theoretical observations.

2 Methods and materials

2.1 Cell plating experiment

The yeast *Saccharomyces cerevisiae* was used as a model organism to analyse plating of cells and colony development. The experiments were performed on circular plates with 88 mm diameter. Sterile 4 mm glass plating beads (manufactured by Sigma-Aldrich) were used for the dispersion of cells over the surface of a plate. Colonies were grown at 30 °C on rich media (Yeast Peptone Dextrose agar) [14].

To quantify the effectiveness of plating with glass beads, the plating was carried out with different concentrations of *S.cerevisiae* cells. The yeast culture was diluted with liquid YPDand 6 serial dilutions (in 1:10 dilution steps) were prepared in sterile media. The last three dilutions with concentrations of 10^2 ppm, 10^1 ppm, and 10^0 ppm, were used for cell plating. Concentration of $1ppm$ corresponds to 1 μl of yeast cultures per 1 l of water.

100 μl of the diluted cell suspension was pipetted on to solid media in plates, then 10 glass beads were added and the plates were agitated. The plates were moved on a trajectory resembling the horizontally reflected letter "L": up-right-left-down. The number of plate movements was varied: either 5 "L" loops or 10 "L" loops. The glass beads were removed by inversion of the plates. Experiments were performed in triplicate.

Plates containing the CFUs were incubated for 72 h at 30 °C. For each plate showing growth, the colony counts were performed manually and later confirmed by tracing yeast colonies on digital images, as shown in Fig.2 (c). This also allowed us to calculate a surface coverage. The numbers of colonies were analysed by two-way ANOVA with Duncan's post hoc multiple comparisons test. The statistically significant level was set to $p = 0.05$.

2.2 Mathematical model

We sought to develop a model that can explain both the origin and the variability of glass beads behavior while plates are agitated. First, we started with a circular billiard which is stationary in space. Without loss of generality, we set the radius of the circle to be 1. A single particle moved without friction between the boundary of the unit circle. Particle movement was defined by the elastic collision rule: the angle of reflection is equal to the angle of incidence [11]. The dynamics of particle movement can be described in terms of collision maps, where θ_k denotes the point of the collision and φ_k denotes the reflection angle for k^{th} collision, $k = 1, .., n$. We adopted a system where $\theta = 0$ corresponds to a point $(1, 0)$ in a Cartesian coordinate system. Figure 1 (a) shows a collision of a particle with the boundary at the point $\theta = \pi$ and $\varphi = \frac{\pi}{3}$. The dynamics of a particle in the circle is fully integrable and is described by the following properties: (i) $\varphi_k = \varphi_1$, i.e. the angle of the reflection does not change; (ii) the coordinates of the point of collision satisfies $\theta_k = (\theta_1 + 2k\varphi_1) \mod 2\pi$; (iii) if $\varphi < \pi$ and is a rational multiple of π, then the particle trajectory is periodic

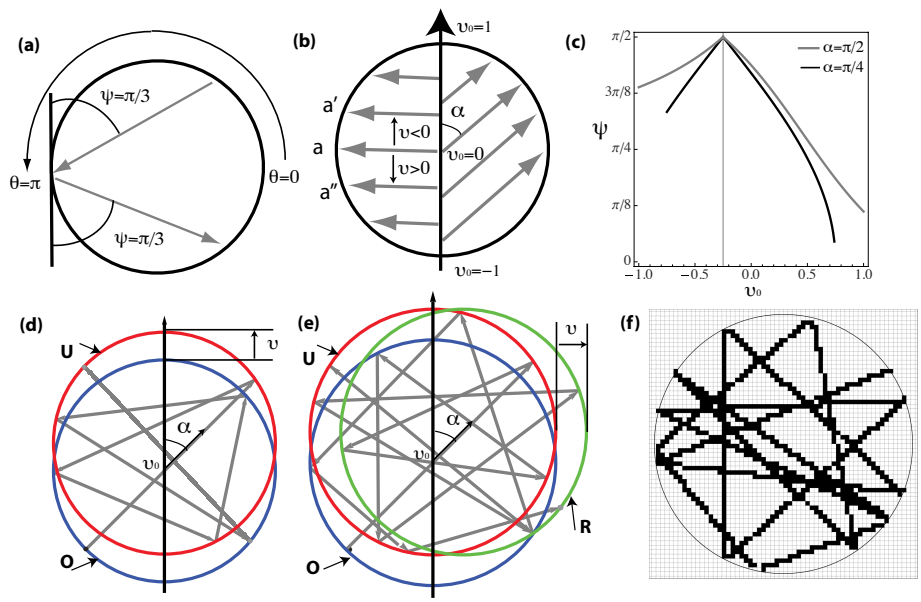

Figure 1: (Color online) Mathematical model and box count: (a) configuration of the system: reflection angle φ and collision point θ; (b) shifted circle: distance of the shift v, angle α and coordinate v_0 ; (c) reflection angle after shift $v = 0.25$; (d) trajectory of the particle for up-down shifting of the circle; (e) trajectory of the particle for up-right-left-down shifting of the circle; (f) box count with $h = 0.022$ for the trajectory in (e).

with the particle following the sides of some regular polygon; (iv) otherwise, if $\frac{\varphi}{\pi}$ is irrational, then the particle trajectory densely fills the ring between boundary of the unit circle and the boundary of a smaller circle with radius $\cos^2(\varphi)$ [10]. In the latter case, there are no trajectories formed inside this smaller circle. For the case (iii), the number of polygon sides depends on the reflection angle. For example, if $\varphi_1 = \frac{\pi}{2}$, the polygon is a square and particle moves in period-4 orbit along sides of the square; and for $\varphi_1 = \pi$ the particle runs forth and back along a diameter of the circle and is locked in period-2 orbit.

Next we introduce a new class of billiard - shifting billiard, which serves as a conceptual abstraction of the cell plating with glass beads. The circle is shifted in such a way that its center moves distance v along the y axis, i.e the coordinates of its center is $(0, v)$. As the circle boundary is moved, the collision point also changes. For a particle moving along the x axis and perpendicular to the y axis, i.e. with $\theta = \pi$ and $\varphi = \pi$, the collision point becomes a' for $v > 0$ (the circle shifted up) or a'' for $v < 0$ (the circle shifted down), as shown in fig.1 (b). We denote by α the angle between the trajectory of the particle and the direction of the circle shift, ($\alpha \in [0, \pi]$). We also denote by v_0 the coordinate where the line going through the trajectory of the moving particle intercepts

the circle shift directory. Graphical representation of α and v_0 is shown in fig. 1 (b). If the angle $\alpha < \frac{\pi}{2}$, this indicates that the particle moves in the same direction as the circle has been shifted. Otherwise, if the angle $\alpha > \frac{\pi}{2}$, then the particle moves in the direction which is opposite to the direction that the circle has been shifted. Potentially, $v_0 \in (-\infty, \infty)$, but not all α-v_0 values will give trajectories contained within the moving circle boundaries. Allowed values of v_0 are within the interval $[v_0^1, v_0^2]$, where $v_0^1 = v - \sin(\alpha)^{-1}$ if $\alpha \leq \frac{\pi}{2}$, $v_0^1 = v - 1$, if $\alpha > \frac{\pi}{2}$; and $v_0^2 = 1$ if $\alpha \leq \frac{\pi}{2}$, $v_0^2 = \sin(\alpha)^{-1}$ if $\alpha > \frac{\pi}{2}$.

As the point of the collision changes, so does the angle of the reflection. Figure 1 (c) shows the reflection angle as a function of v_0 for two values $\alpha = \frac{\pi}{2}$ and $\alpha = \frac{\pi}{4}$ after the circle has been shifted up by $v = 0.25$. For both values of α, the reflection angle is equal to $\frac{\pi}{2}$ for $v_0 = -v$ and decreases for other values of v_0.

After the first collision, the circle will return back to its original position with center located at (0,0). Dynamics for the particle moving in the shifting circle are shown in fig. 1 (d). Initial conditions for the particle are $v_0 = 0$ and $\alpha = \frac{\pi}{4}$, and the circle is shifted by $v = 0.25$. Here **O** indicates the original position (blue circle online) and **U** - the "up" position (red circle online). For this particular set of the parameters, the particle is fixed in a period-2 orbit after 6 collisions the with boundary of the circle.

Finally, we introduce a more complex movement of the circle. The circle is shifted according to the following loop: it is moved up (the "up" position, **U**), right (the "right" position, **R**), left (returned to the **U** position), and down (return to the **O** position). Figure 1 (e) shows the trajectory for a particle with the same initial conditions as in 1 (d), only this time it breaks away from period-2 orbit after the circle is shifted to the position **R**.

2.3 Calculating box count

The box count was defined as a ratio between the number of squares which contain part of a glass bead trajectory and the total number of the squares. We discretized an area of the plate into equally sized squares. We denoted the side of square to have length h. If a glass bead moves in a line between any two points on the plate, we expect that the substrate cells can be spread anywhere in the rectangle with area equal to the length of a line multiplied by radius of the bead. Therefore we set $h = 0.022$ units, which is the ratio between radius of a bead to the diameter of the plate, giving 6490 boxes per plate.

The trajectory of the particle in shifting circles has been merged into a single trajectory in a circle. Figure 1 (f) shows a discretisation of the circle surface and merged trajectory given in fig. 1 (e). The trajectory is plotted only after the first collision with the circle boundary, since initial condition were not fixed, only the direction of initial movement. The box count for the trajectory is 0.18.

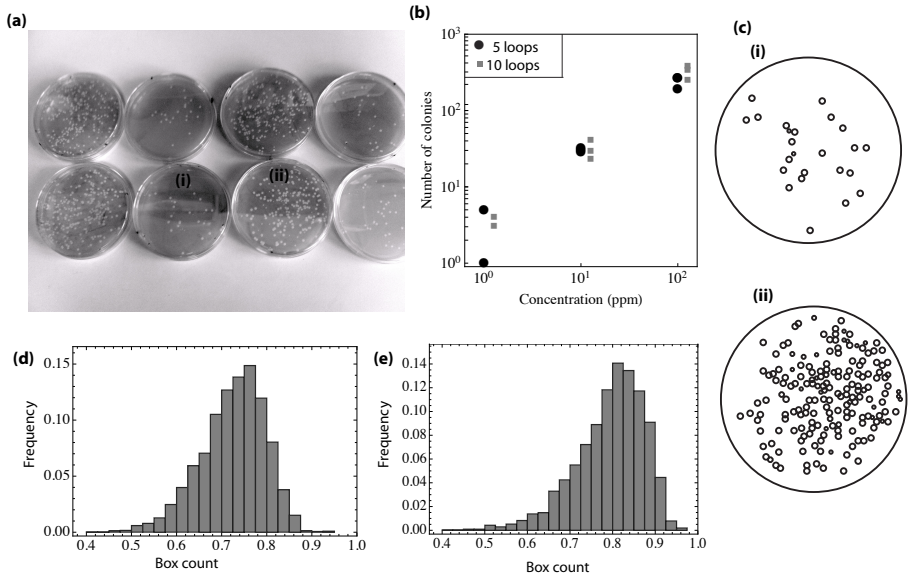

Figure 2: Results: (a) grown colonies after 72h incubation period; (b) the number of colonies as a function of the concentration and the plating method: 5 loops (black circles) and 10 loops (gray squares); (c) the digitised versions of the plates (i) and (ii) in (a); (d) frequency distribution for the box counts for 5 loops; (e) frequency distribution for the box counts for 10 loops.

3 Results

Figure 2 (a) shows colony growth after 72h incubation for selected plates. For a higher concentration we observed a good distribution of colonies in all three replicas. For the concentration of $10^0 pmm$, one out of three plates had no viable colonies for both configurations. Figure 2 (b) shows the number of isolated colonies as a function of concentration and plating configuration. The number of colonies were counted twice: manually 72h after incubation and when analysing the digitised versions of the plates (Fig. 2 (c)). Both methods gave the same results. Analysis of variance for the number of colonies indicated statistically significant differences between concentration of yeast cells ($p = 9.6 \times 10^{-14}$), but no statistically significant differences between number of loops performed for plating (p=0.15). A post-hoc Duncan's test found that the number of colonies for the concentration $10^2 pmm$ and $10^0 ppm$ were significantly different from number of colonies for the concentration $10^1 ppm$ at $p = 1.2 \times 10^{-8}$, as would be expected. Surface coverage was quantified as the ration between area covered by colonies (small circles in Fig. 2 (c)) to the area of the plate. Analyses of the digitised versions of the plates gave that the average surface coverage of the colonies for 5 loops and 10 loops was 0.24 and 0.29 for the concentration $10^2 ppm$.

We used the mathematical model described in Section 2.2 to simulate plating with glass beads. The number of glass beads was fixed at 10 for each run. We extended the model for multiple particles but enforced the following assumptions: (i) glass beads move freely without friction on the surface of the plate; (ii) the plate moves according to the loop: origin (**O**), up (**U**), right (**R**), left (**U**), and down (**O**); (iii) whenever a glass bead can not freely move along its reflected trajectory, the trajectory is terminated; (iv) an interaction between glass beads is presumed to have a negligible effect on the dynamics (ten glass beads occupy less then 1.2% of plate area).

The initial conditions, α and v_0, were selected randomly for each particle. The trajectory of each particle was followed either for 5 loops of movement or for 10 loops of movement. The trajectories of all particles after the first collision with the circle boundary have been merged into the single circle and the box count performed as described in Section 2.3. Figure 2 (d) and (e) shows a frequency distribution of box count for $v = 0.25$, run for 10^4 simulations. The proportion of the plate area covered by 5 "L" loops is from 0.42 to 0.94, and by 10 "L" loops is from 0.42 to 0.97. The mean of box count was 0.73 for 5 "L" loops and 0.79 for 10 "L" loops. Pairwise analysis for the same initial conditions gave that the additional 5 loops will increase the box count by 0.06 on average.

The summary of the results is given in Table 1.

4 Discussion

We studied culture plating and subsequent colony growth by performing experiments with the yeast *S.cerevisae* and by simulating the dynamics of the particle in a shifting billiard model. Plates with glass beads were agitated by moving in a loop up-right-left-down pattern with the number of loops either 5 or 10. We used a similar set up for the numerical simulations of the mathematical model and characterized the output in terms of box counts.

Caution should be taken when directly comparing simulation outputs to experimental results. First, although all trajectories travelled by beads are continuous, the trail of cell suspension left behind a bead may be discontinuous. Secondly, the success in the establishment of colonies depends on different factors such as the quality of medium nutrients, growth kinetics, incubation time (surface coverage after 36h will be less then that after 72h because colonies will be smaller) or quorum sensing [15]. Thirdly, and most importantly, the number

Table 1: Number of colonies and surface coverage for the concentration $10^2 ppm$ and the simulated box count, shown as mean (standard deviation).

	Experiment		Simulation
Method	Number of colonies	Surface coverage	Box count
5 loops	242 (44.1)	0.24 (0.04)	0.73 (0.07)
10 loops	323 (70.4)	0.29 (0.06)	0.79 (0.07)

of viable cells that are dispersed in the cell suspension is a random variable, this will produce a variation between experimental replicas, as well as between the two methods. Therefore, the experiment and simulation should complement each other in their findings when evaluating the method.

Statistical analysis of the number of colonies grown after 72h of incubation has not shown significant differences between the number of colonies plated using 5 or 10 loops. The simulation based on the mathematical model also indicated that the distribution of box counts for both configurations was very similar. On average, trajectories formed by 5 loops covered 73% of the plate surface, and trajectories formed by 10 loops covered 79% of the plate surface (Table 1), which on average gave an extra surface coverage of about 6%. For cell suspension with concentration $10^2 ppm$ the average surface coverage of the colonies was 24% for 5 loops and 29% for 10 loops (Table 1), which gives a difference in the average surface coverage of 5%.

In conclusion, our results indicate that the Copacabana method is highly efficient and, if used with an appropriate dilution factor, will yield optimal spreading of the substrate. When the plate is moved in the trajectory resembling an L-shape, as little as 5 loops will be sufficient to have appropriate colony separation, which should save time, especially when running an experiment on multiple plates.

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