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In vitro microbial culture models and their application in drug development☆

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ABSTRACT

Drug development faces its nemesis in the form of drug resistance. The rate of bacterial resistance to antibiotics, or tumor resistance to chemotherapy decisively depends on the surrounding heterogeneous tissue. However, in vitro drug testing is almost exclusively done in well stirred, homogeneous environments. Recent advancements in microfluidics and microfabrication introduce opportunities to develop in vitro culture models that mimic the complex in vivo tissue environment. In this review, we will first discuss the design principles underlying such models. Then we will demonstrate two types of microfluidic devices that combine stressor gradients, cell motility, large population of competing/cooperative cells and time varying dosage of drugs. By incorporating ideas from how natural selection and evolution move drug resistance forward, we show that drug resistance can occur at much greater rates than in well-stirred environments. Finally, we will discuss the future direction of in vitro microbial culture models and how to extend the lessons learned from microbial systems to eukaryotic cells.

Contents

1. Introduction

The emergence of antibiotic resistance in bacteria remains a persistent problem worldwide [1]. Previous studies isolated and characterized various resistant mutants, which provided valuable insights into the biological processes that are altered in mutant bacteria [2–4]. But the rate of evolution of antibiotic resistance is still unclear, especially in an environment that bacteria naturally live. Understanding the rate of evolution is crucial for developing new antibiotics and planning effective treatments.

Microfluidics is the science and technology of systems that process or manipulate small (10^{-9} to 0^{-18} l) amounts of fluids [5,6]. Microfluidics provides a reproducible and controllable way to reconstruct various important factors of in vivo environments, which is challenging to achieve via conventional test tubes [5,7,8]. Two major types

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of control have been realized in microfluidic devices. One is controlling the reaction inside the device via the laws of fluids at the small scale. Our group pioneered this approach by demonstrating rapid mixing in a hydrodynamic focusing device [9] and further added additional passive controls by creating regular structures on silicon wafers [10–13]. The other approach of the control is to make on-chip active components — valves [14–16], mixers [17–19] and pumps [20,21]. Using active or passive control, many methods to culture bacteria in microfluidic systems have been published [22–26,7], including recent microfluidic devices to mimic bacterial growth in human tissue [27,28].

When members of a bacterial community are allowed to interact with complex environments, dramatic changes in the pace of evolution and selection have been seen in both experiments and simulations [29–33]. Specifically, ecological conditions like drug gradients and the presence of small population niches can induce rapid drug resistance [34,35,10]. Thus, full understanding of the nature of drug resistance requires controlling these ecological conditions along with cell culture. Here, we will review two main types of microfluidic devices recently developed as in vitro microbial culture models combined with ecologies. We will highlight the key results got from experiments performed by combining these new devices with other technologies in genomics such as next generation sequencing and DNA microarray. Finally, we will provide an outlook on two aspects: one is the advancement of in vitro microbial culture models in the future, particularly how microfluidic devices can be integrated with various genomics analysis tools as a unified platform to assess the development of drug resistance in a high-throughput and systematical fashion; the other is how to apply knowledge learned from microbial systems to eukaryotic systems, where both opportunities and challenges will be discussed.

As an aside, applications of microfluidics to drug development have been well reviewed [36,37], and in particular, there have been many rapid developments in using droplet based approaches for economically useful mutants [38]. We refer the reader interested in drug development to those reviews and papers. In this review we shall only focus on the coming together of cell culture and ecological niches to influence drug resistance in microfluidic devices.

2. Wright's evolution principle

The design principles of the new generation of in vitro microbial culture models came from Wright's seminal contributions concerning the importance of the number of cells n_i in a particular microhabitat i [39,40]. The number n_i of individuals within a particular microenvironment niche strongly influences the outcome of natural selection on the fixation probabilities $P(n_i,s_i)$ and fixation times $\tau(n_i,s_i)$ within that particular microhabitat. Suppose that in a population of n_i cells, a mutation appears in an individual with a relative fitness advantage s_i over the other $n_i - 1$ competitors. The probability due to genetic drift for the eventual fixation of that mutation in all the n_i individuals scales as:

$$P(n_i,s_i) = \frac{1-exp^{-2s_i}}{1-exp^{-2n_i s_i}} \quad (1)$$

while the mean time to fixation scales as:

$$\tau(n_i,s_i) \sim \frac{1}{s_i} ln(n_i). \quad (2)$$

Eqs. (1) and (2) have some interesting implications. First, note the explicit dependence on the number of competing individuals n_i, so that the probability of fixation is dependent on the product of the fitness s_i and the number of competitors $n_i - 1$. In a large population, which means that $s_i n_i \gg 1$, the most fit clone *always* wins and fixes the mutation. However, if $s_i n_i \leq 1$, it is entirely possible for less fit clones to fix, even clones with negative fitness! Fig. 1 shows the dependence of the probability of fixation on population number n_i and relative fitness s_i. This is an important point: what really matters in fixation probabilities is the product of fitness times population number. Of course, this formula is just for genetic drift in a population and does not describe true evolution which involves both the generation of mutations and the process of natural selection, but it should give some idea of the role that population size plays in the role of gene fixation. Similarly, the time to fixation also depends on the number of competitors: the larger n_i is, the longer it takes to fix.

Within a complex ecology such as a biofilm we can expect that there is spatial and temporal heterogeneities in the fitness s_i and number n_i as long as there is an initial mutagenic aspect to the cell reproduction. Also, because of the complexity of a biofilm or bacteria within the tissue, there will be very strong gradients in drug delivery, with a corresponding distribution in the probabilities of fixation and time to fix for mutants which show the phenotype of resistance.

Little is known about the influence of the steepness of fitness gradients on the rates of evolutionary adaptation to stress, but we can guess a few things. First, recall from Eq. (1) that the smaller the number of cells at a given fitness advantage, the more likely fixation is to occur. On the other hand, there is also the phenomenon of Muller's Ratchet [41], which is that the smaller the population, the more likely it is for lower fitness mutants to fix, if there is no exchange from other populations. Thus, it is difficult at present to truly predict what is the optimum drug dose without detailed knowledge of the drug gradients, metapopulation sizes and motility between local populations. However, if these can be measured then a true theory of evolution of drug resistance evolution rates is within reach.

3. Microfluidic devices implementing spatial drug gradients

3.1. Rapid evolution in microfluidic devices

Our fundamental point concerning the inevitable emergence of resistance to a mutagenic stressor under the appropriate ecological and metapopulation parameters can be shown by designing using microfabrication techniques designed to quantitatively test these ideas. We have done a fundamental experiment which shows the power of these ideas [10]. The design and characterization of the devices are shown in Fig. 2.

We used *Escherichia coli* bacteria and the highly mutagenic bacteriastatic antibiotic ciprofloxacin. Ciprofloxacin is a member of the quinolone family of antibiotics and functions by binding to DNA gyrase [42]. Ciprofloxacin traps the gyrase–DNA complex at the state when the DNA is cut, thereby inhibiting DNA replication and cell division, in essence preventing the cell from dividing but not killing the cell (i.e., it is cytostatic, not cytotoxic). The generation of single-stranded DNA by stalled ciprofloxacin-bound gyrase is known to trigger, via the self-cleavage of the repressor LexA, removal of LexA from transcription factor sites. Removal of LexA activates the transcription error-prone DNA polymerases [43]. The effective mutagenic rate u^* due to the SOS response is 10^{-5} mutants/viable cell/day, 10,000 times greater than the base rate u [44].

Fig. 3 shows the emergence of resistance from wild-type *E. coli* over 20 h. When bacteria are inoculated into the center of the device, chemotaxis due to consumption of nutrients at low flow rates quickly drives them to the perimeter of the device. At the Goldilocks point (gold arrow, Fig. 3(A)) there is a combination of high population gradient and high mutation rates. In this experiment the concentration of ciprofloxacin flowing along the bottom side of the device is 10 μg/mL, approximately 200 times the minimum inhibitory concentration of ciprofloxacin. Yet, as Fig. 3 shows there is ignition of resistance at the Goldilocks point and subsequent rapid movement of resistant bacteria around the periphery of the device and invasion back to the center in 20 h.

The basic reason for this invasion of resistance is the fitness advantage for mutant resistant *E. coli* in a micro-environment where the