INSTITUTE OF PHYSICS PUBLISHING JOURNAL OF MICROMECHANICS AND MICROENGINEERING

J. Micromech. Microeng. **15** (2005) N6–N10 doi:10.1088/0960-1317/15/1/N02

TECHNICAL NOTE

Theoretical considerations for counting nucleic acid molecules in microdevices

Madhavi Krishnan[1,4], **David T Burke**[2] **and Mark A Burns**[1,3]

[1] Department of Chemical Engineering, The University of Michigan, Ann Arbor, MI, USA
[2] Department of Human Genetics, The University of Michigan, Ann Arbor, MI, USA
[3] Department of Biomedical Engineering, The University of Michigan, Ann Arbor, MI, USA

E-mail: maburns@umich.edu

Received 14 July 2004
Published 15 October 2004
Online at stacks.iop.org/JMM/15/N6

Abstract

We describe the theoretical design of a microchip for the amplification, detection and counting of individual nucleic acid molecules in an ensemble of non-specific molecules. The device permits access to individual molecules through the transformation of a three-dimensional amplification reaction volume into a two-dimensional area or a one-dimensional line. The nucleic acid molecules in the amplification solution can be spatially separated along a one-dimensional line or across a two-dimensional area. The arrangement of the molecules is governed by the geometry of the microreactor in a manner analogous to the concept of 'limiting dilution' in a volume of fluid. When PCR amplification is allowed to proceed, the target sequences on individual molecules are amplified and diffuse outward. At the end of the amplification process, the zones of concentrated DNA surrounding the individual molecules can be detected as spatially resolved signals through the use of DNA binding dyes or fluorescence resonance energy transfer (FRET) probes. These amplification peaks would serve to establish a count of the number of target molecules in a sample with a much higher accuracy than that of traditional quantification using real-time PCR. The theoretical accuracy of this technique is only limited by the Poisson statistics of sampling.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Quantifying a specific population of nucleic acid molecules amid an ensemble of non-specific molecules is an important analytical technique in molecular biology. The application of nucleic acid quantification ranges from gene expression studies which focus on quantifying the number of mRNA molecules in a sample to clinical diagnostics for the detection and quantification of viral and bacterial DNA. Conventional laboratory bench-scale nucleic acid copy number estimation techniques, such as real-time PCR, are able to reliably provide order of magnitude estimates of the number of molecules in a sample [1, 2]. This lack of accuracy may be attributed in part to the fact that the progress of the reaction is monitored in a three-dimensional volume, resulting in an amplification signal from reaction kinetics averaged over the entire reaction volume. The three-dimensional nature of the assayed volume also obscures the individual molecules from view during the amplification process, making them inaccessible for counting as individual molecules, and lowering the sensitivity of the counting process. An additional limitation on the accuracy of real-time quantitative PCR is the fact that the technique relies on the comparison of the amount of PCR product generated or the amplification kinetics of the unknown sample with that of

[4] Present address: Institut für Biophysik/BioTec, Technical University, Dresden, Germany.

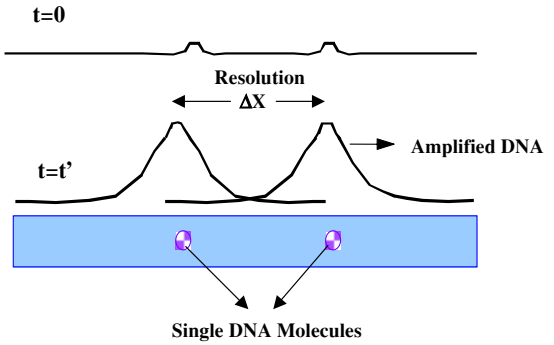


Figure 1. Schematic illustrating the concept of spatial separation of individual molecules in one- or two-dimensional space. At time $t = 0$, the PCR solution is loaded into the microstructure and the individual molecules are spatially separated by a distance Δx. At the end of the PCR amplification process ($t = t'$), the zones of amplified DNA molecules surrounding the target molecules may be detected using fluorescence through the incorporation of DNA binding dyes or FRET probes in the reaction. The distance Δx between the target molecules determines the ability to detect them as spatially resolved peaks of amplified DNA.

a known standard that is biochemically similar but not always identical to the system under quantification [3, 4].

We describe the theory and design of a microdevice to accurately count the number of nucleic acid molecules in an ensemble of non-specific molecules through nucleic acid amplification. The principle of this microdevice is based on the transformation of the reaction space from a three-dimensional volume to a two-dimensional area or a one-dimensional line. This transformation of the reaction space results in the spatial separation of molecules that can now be monitored individually during the course of DNA amplification. The diffusivity of DNA in an aqueous solution results in higher local concentrations of the amplified segment of DNA in the vicinity of each target molecule compared to the background. DNA binding dyes or FRET probes incorporated into the amplification reaction would permit these concentrated zones of DNA to be detected as local regions of enhanced signal over a lower background [5]. The number of concentrated zones detected would be theoretically equal to the number of single molecules loaded into the device. The process would involve minimal post-PCR processing compared to previously described systems [6, 7]. It is also possible to construct quantification systems where the reaction space is physically divided into a number of individual cells that are interrogated for the presence or absence of target molecules in a nanoarray. This technique involves the use of barriers to physically or chemically divide the reaction zone into separate cells instead of the physics of dimensionally restricting diffusion to spatially separate individual parent molecules.

2. Theory and principle

Numerical simulations were performed using MATLAB to determine the time-dependent DNA concentration profile around molecules being amplified in a microchannel (figure 1). PCR kinetics were modeled as an exponential increase in DNA concentration, with a doubling time equal to an estimate of the cycle time (about 60 s). The model description did not

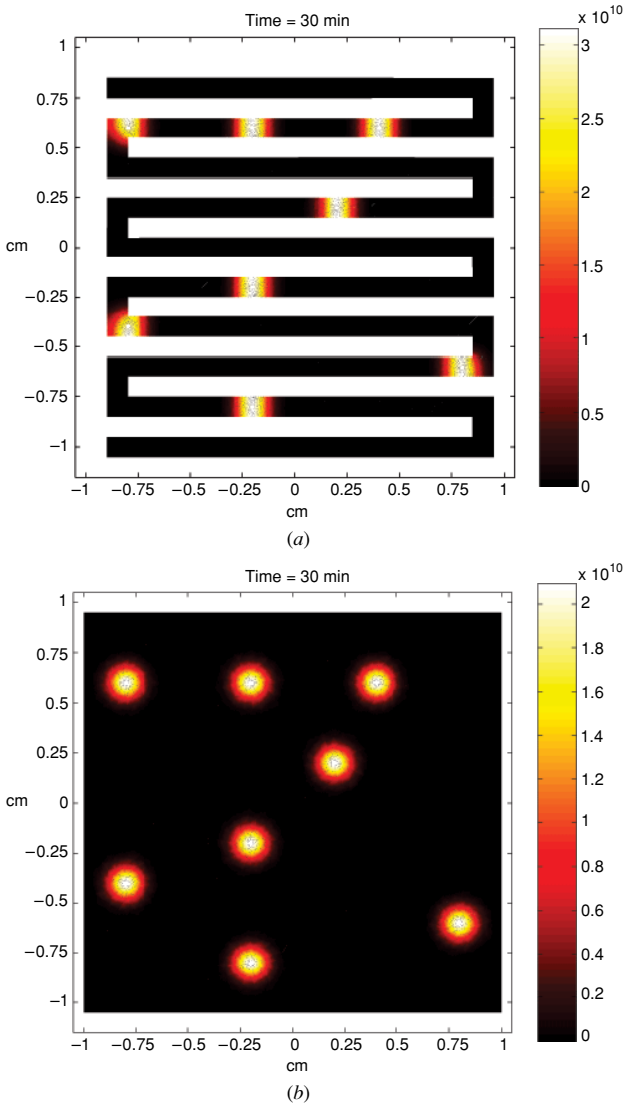
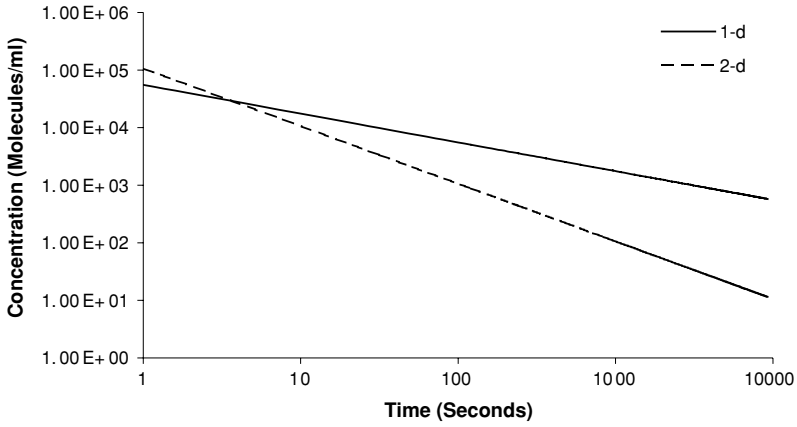


Figure 2. Simulations of concentration profiles of amplified DNA surrounding identical distributions of target molecules in one- and two-dimensional systems, in a device with an area of cross-section of 1 cm^2. At low concentrations, the target molecules are separated well enough for the surrounding amplification zones to be detected as resolved peaks in both systems. Note that, since the one-dimensional case (*a*) contains the same number of molecules as the two-dimensional case (*b*) in half the area/volume, the solution in (*a*) is actually twice as concentrated as (*b*).

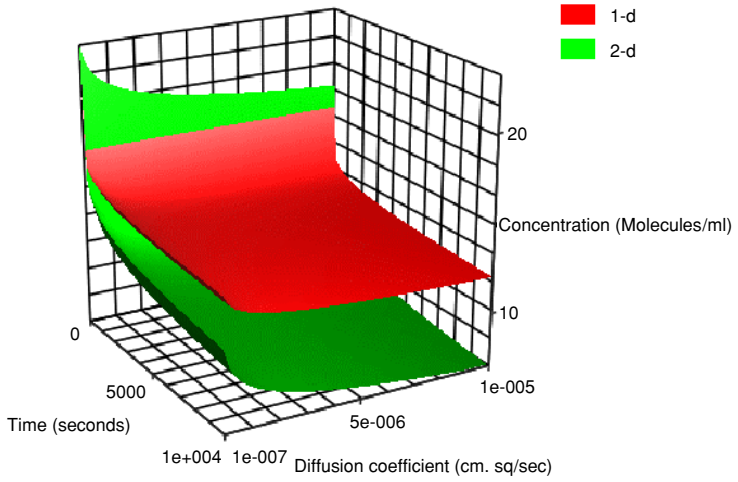
incorporate the saturation kinetics of PCR amplification [8], but this is not expected to drastically affect the concentration profiles surrounding the molecules; in fact, incorporation of the saturation kinetics would be expected to have a beneficial effect in restricting the width of signal peaks surrounding the amplified target molecules. The system can be described by an unsteady-state diffusion model with exponential generation in two dimensions:

$$\frac{\partial C}{\partial t} = D\left(\frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2}\right) + kC$$

where k is a first-order rate constant for PCR and was estimated as $(\ln 2)$/cycle time or around 0.01 s^{-1}. The initial condition



(*a*)



(*b*)

Figure 3. Plots representing the variation of the concentration of molecules that can be successfully resolved and counted in one- and two-dimensional systems with time of amplification (*a* and *b*) and diffusion coefficient of the diffusing species (*b*). The number of molecules that can be resolved and counted was estimated using a uniform distribution of target molecules over the reaction space. The volume of the structures used to determine concentrations was estimated assuming the depth of the microstructure to be 30 μm. (*b*) The graph represents a three-dimensional plot of the functions depicted in (*a*) but includes variations with the diffusion coefficient of the diffusing species which, in this case, is the amplified DNA fragment.

used was $C = 0$ at $t = 0$ and at the physical boundaries of the microchannel the flux of molecules was zero: $\left(\frac{dC}{dx}, \frac{dC}{dy} = 0\right)$. The transient problem was solved numerically using the finite element method.

3. Results and discussion

3.1. Simulations and comparison of one- and two-dimensional counting systems

Transient simulations of DNA diffusing in a microchannel with simultaneous exponential generation were performed. An arbitrary starting configuration of single molecules was simulated in a microreactor in order to obtain an estimate of the number of molecules detectable through the formation of discrete amplification zones as a function of factors such as channel geometry and reaction time. The diffusion coefficient of DNA used in the simulations was an average diffusion coefficient for a 300 bp fragment of dsDNA over the range of PCR temperatures and was of the order 10^{-6} cm^2 s^{-1}. The inclusion of FRET probes or DNA binding dyes such as SYBR Green in the PCR mix would enable direct *in situ* counting of molecules in the chip with minimal post-processing. FRET probes are about 25 bp in length and the diffusion coefficient of a double stranded DNA fragment of that size of the order of 10^{-6} cm^2 s^{-1} [9]. The incorporation of DNA binding dyes and the labels attached to the FRET probes would have a beneficial effect in restricting the diffusion of the amplification zones surrounding the target molecules.

Two types of reactor geometries were considered in the simulations and are shown in figure 2. A comparison of the 'counting resolution' (number of discrete amplification zones/device area) in one- and two-dimensional reaction systems for a given device area and a uniform distribution of target molecules over the reaction space is shown in figure 3.) The plot for the one-dimensional case is given by $\frac{1}{V}\left(\frac{L}{2\sqrt{Dt}}\right)$ where L is an estimate of channel length that could be conveniently incorporated in a device of area 1 cm^2 and V is an estimate of the volume of fluid in the microchannel (the dimensions assumed for such a one-dimensional channel were:

length = 20 cm, width = 30 μm and depth = 30 μm). The plot for the two-dimensional case is given by $\frac{1}{V}\left(\frac{A}{\pi Dt}\right)$ where A is the area of the reaction space, 1 cm^2, and V is the volume of fluid in the microchannel, assuming a depth of 30 μm. For a given device size, the two-dimensional system occupies a larger effective area than the one-dimensional system. Therefore, at the onset of amplification, when the zones of concentrated DNA surrounding the target molecules are very small, the larger effective area of a two-dimensional system results in an apparently higher 'counting resolution' for this system. However, on time scales comparable with that of the PCR process (about 30 min for most microchip systems [10, 11]), the zones of amplified DNA surrounding each parent molecule get larger, and the overlap of gradients in two dimensions progressively degrades the resolution of the two-dimensional system compared to the one-dimensional case (figure 3). Thus the simulations indicate that from a theoretical perspective, the one-dimensional reactor configuration would be a more effective counting platform than the two-dimensional area format on the time scale of PCR amplification.

3.2. One-dimensional counting systems

The simulations show that the dimension of the zone of high-fluorescence intensity surrounding the amplifying parent molecule over the time scale of the amplification process is about 2.5 mm. This result implies that a device incorporating a microchannel length of about 100 mm could theoretically be used to count up to 40 individual molecules. The cross-sectional area of the microchannel used for counting would depend on the concentration of target molecules in the sample. In general, channels with smaller cross-sectional areas would be more suitable to the counting of higher target concentrations; a smaller volume of fluid would be stretched out to a longer effective length resulting in a greater linear separation of the target molecules. As the height and width of the channels can be fabricated to dimensions as small as 0.2 μm using conventional photolithographic and microfabrication techniques, it would be possible to fabricate channels to match a wide range of concentrations under interrogation.

An interesting modification of this format is the case of a microchannel with a decreasing cross sectional area (figure 4(*b*)). This design is, in effect, the adaptation of the concept of 'limiting dilutions' to the microchip format. For a sample of given concentration loaded into the channel, the target molecules would be closely packed in regions of the microchannel where the cross-sectional area is high. Therefore, the amplification signals from the individual molecules would overlap with each other and distinct peaks from amplification of these molecules would not be discernible (figure 4(*a*)). As the cross-sectional area decreases, the target molecules in the sample undergo the process of dilution along a one-dimensional line. Eventually, the sample would occupy a section of the channel where the area of cross-section ensures detection of the amplification zones. Figure 4(*b*) shows the effect of this dilution using 1000, 500 and 250 μm wide channels. Thus, a single chip could be used to accurately quantify the number of target molecules in samples spanning a wide range of concentrations.

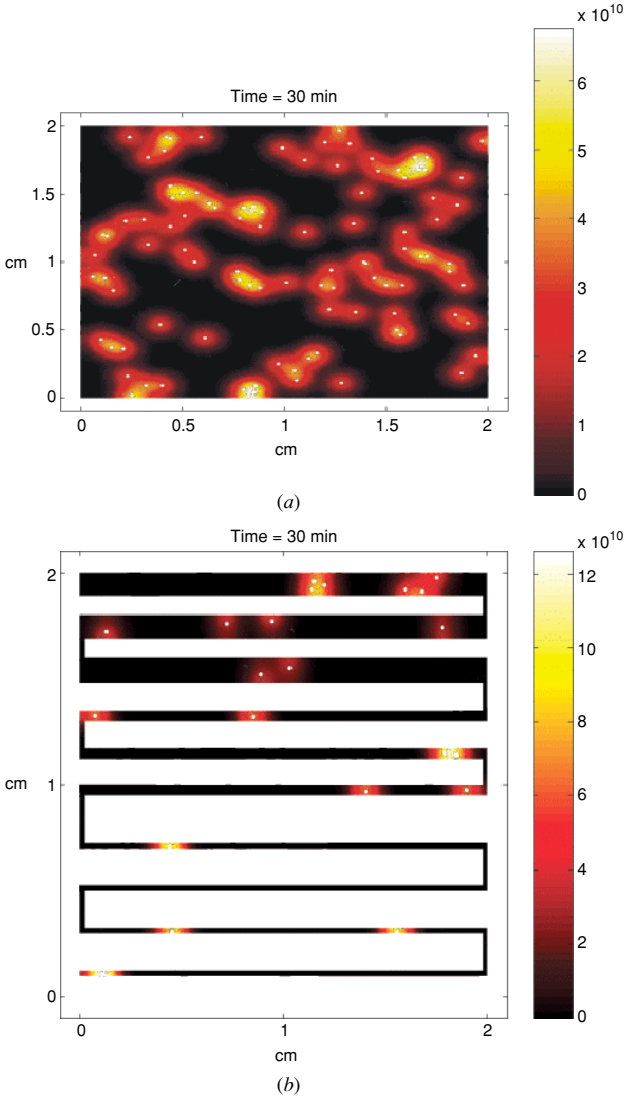


Figure 4. Comparison of 'counting efficiency' of an identical concentration of target molecules in one- and two-dimensional systems. An identical configuration of randomly generated distribution of target molecules was used in both cases. (*a*) This simulation shows the two-dimensional concentration profiles surrounding a random distribution of 100 target molecules in a 400 mm^2 area. The simulations clearly show an overlap of amplification zones in the vertical dimension in the two-dimensional system that could result in a poorer 'counting resolution' in this case compared to that of the one-dimensional system in (*b*). (*b*) This simulation demonstrates the effect of decreasing width or area of cross-section of the reaction space on counting resolution in a one-dimensional system. Starting from the top, the first three lanes are 1mm wide, the next three lanes are 500 μm wide and the last four lanes are 250 μm wide. A decreasing channel cross-sectional area has the effect of lowering the density of distribution of target molecules per microchannel length in a sample of given concentration. This effect is borne out in the simulation which shows overlapping amplification zones in sections of the microchannel with a large area of cross-section, and therefore a high density of target molecules, and discrete zones in sections of channel with a smaller cross-section, and therefore lower density of target molecules. Note that the expected number of molecules in each section (based on a concentration of 100 molecules/ 400 mm^2) is close to the actual: 15, 7.5 and 5 versus 12, 7 and 4.

4. Conclusions

The microdevice described in this note presents an elegant platform to perform quantitative PCR on a chip. The device is based on the adaptation of the principle of 'limiting dilution' to the microchip format. The molecules are diluted along a one-dimensional line, amplified using PCR and counted using end-point detection. Each discrete linearly separated peak corresponds to a single parent DNA molecule. The simple underlying physics overcomes the fundamental limitations of real-time quantitative PCR and enables the goal of counting to be achieved with a much higher counting resolution. The device modeled and described in this work could present an opportunity for significant operational advancement over many conceptually similar alternatives by completely eliminating the need for post-PCR sample processing. In addition to being compatible with mass production by microfabrication techniques, the device could be adapted to a completely self-contained, low-power, portable, integrated system.

An important practical consideration in the operation of the single molecule counting device described here however, involves ensuring the efficiency of PCR amplification while scaling the microchannel down to dimensions compatible with quantification of copy number in concentrated samples. As the surface-to-volume ratio increases dramatically during the transformation of a three-dimensional reaction space to a one- or two-dimensional structure, the choice of surface materials in microchannel construction can be a critical issue in determining the efficiency of the amplification process, with adsorption of DNA, enzyme and reagents being the primary concern.

In addition to being used as a nucleic acid quantification device, the functionality of the device may be extended through the incorporation of electrodes on the base of the microchannel. The electrodes would enable the trapping of an enriched population of nucleic acid molecules amplified from a single target molecule for further manipulation and analysis [12, 13], in a manner that is analogous to bacterial colony culture in a petri dish. The physics of this system could also be exploited in studies on other single molecules such as enzymes, where the principle of dilution in one dimension and subsequent observation of single molecule kinetics can be used to yield interesting information on an ensemble of molecules, one molecule at a time [14].

Acknowledgment

This work was supported by the National Institutes of Health under grant P01-HG001984.

References

[1] Lockey C, Otto E and Long Z F 1998 Real-time fluorescence detection of a single DNA molecule *Biotechniques* **24** 744–6
[2] Wittwer C T, Herrmann M G, Moss A A and Rasmussen R P 1997 Continuous fluorescence monitoring of rapid cycle DNA amplification *Biotechniques* **22** 130–8
[3] Sninsky J J and Kwok S 1993 The application of quantitative polymerase chain reaction to therapeutic monitoring *AIDS* **7** S29–34
[4] Gilliland G, Perrin S, Blanchard K and Bunn H F 1990 Analysis of cytokine messenger-RNA and DNA-detection and quantitation by competitive polymerase chain-reaction *Proc. Natl Acad. Sci. USA* **87** 2725–9
[5] Kalinina O, Lebedeva I, Brown J and Silver J 1997 Nanoliter scale PCR with TaqMan detection *Nucl. Acids Res.* **25** 1999–2004
[6] Li H L, Xue G and Yeung E S 2001 Selective detection of individual DNA molecules by capillary polymerase chain reaction *Anal. Chem.* **73** 1537–43
[7] Mitra R D, Butty V L, Shendure J, Williams B R, Housman D E and Church G M 2003 Digital genotyping and haplotyping with polymerase colonies *Proc. Natl Acad. Sci. USA* **100** 5926–31
[8] Liu W H and Saint D A 2002 Validation of a quantitative method for real time PCR kinetics *Biochem. Biophys. Res. Commun.* **294** 347–53
[9] Lapham J, Rife J P, Moore P B and Crothers D M 1997 Measurement of diffusion constants for nucleic acids by NMR *J. Biomol. NMR* **10** 255–62
[10] Belgrader P, Benett W, Hadley D, Richards J, Stratton P, Mariella R and Milanovich F 1999 PCR detection of bacteria in seven minutes *Science* **284** 449–50
[11] Woolley A T, Hadley D, Landre P, deMello A J, Mathies R A and Northrup M A 1996 Functional integration of PCR amplification and capillary electrophoresis in a microfabricated DNA analysis device *Anal. Chem.* **68** 4081–6
[12] Forster A H, Krihak M, Swanson P D, Young T C and Ackley D E 2001 A laminated, flex structure for electronic transport and hybridization of DNA *Biosensors Bioelectron.* **16** 187–94
[13] Lin R S, Burke D T and Burns M A 2003 Selective extraction of size-fractioned DNA samples in microfabricated electrophoresis devices *J. Chromatogr.* A **1010** 255–68
[14] Xue Q F and Yeung E S 1995 Differences in the chemical reactivity of individual molecules of an enzyme *Nature* **373** 681–3