In situ localized amplification and contact replication of many individual DNA molecules

Robi D. Mitra[1,2] and George M. Church[2,*]

[1]Massachusetts Institute of Technology, Department of Electrical Engineering, Boston, MA 02139, USA and
[2]Harvard Medical School, Department of Genetics, Boston, MA 02115, USA

ABSTRACT

We describe a method to clone and amplify DNA by performing the polymerase chain reaction (PCR) in a thin polyacrylamide film poured on a glass microscope slide. The polyacrylamide matrix retards the diffusion of the linear DNA molecules so that the amplification products remain localized near their respective templates. At the end of the reaction, a number of PCR colonies, or 'polonies', have formed, each one grown from a single template molecule. As many as 5 million clones can be amplified in parallel on a single slide. If an Acrydite modification is included at the 5′ end of one of the primers, the amplified DNA will be covalently attached to the poly-acrylamide matrix, allowing further enzymatic manip-ulations to be performed on all clones simul-taneously. We describe techniques to make replicas of these polony slides, and high throughput sequencing protocols for this technology. Other applications are also discussed.

INTRODUCTION

We are faced with an ever increasing demand for DNA sequence information. Currently, most DNA sequencing is done using the Sanger method (1), which relies on electro-phoresis, a technique that is difficult to perform in a highly parallel (>1000 samples at a time per instrument) fashion. Therefore, other technologies are being investigated, such as sequencing by hybridization (2–4) and pyrosequencing (5), that avoid the electrophoresis step of the Sanger method, allowing more samples to be sequenced in parallel. This parallel-ization will increase the throughput of the sequencing stage; however, the samples must first be cloned, amplified and purified, and the throughputs of these obligate stages are not increased by these technologies and remain the major bottlenecks. To address this important issue, we propose a strategy in which a number of samples can be cloned, amplified and sequenced on a single glass microscope slide in a highly parallel fashion.

We have developed a method to deposit DNA directly onto a solid surface and amplify it *in situ*. To do so, acrylamide is polymerized in a solution containing standard polymerase chain reaction (PCR) reagents and a very low concentration of linear DNA template. The gel is poured on a glass microscope slide which is then thermal cycled. As the amplification reaction proceeds, the products remain localized near their respective templates, so that at the end of the reaction, a single template molecule gives rise to a PCR colony, or 'polony', consisting of as many as 10^8 identical DNA molecules. By including an Acrydite modification (6) on the 5′ end of one of the PCR primers, the amplified DNA in each polony can be covalently attached by one of its ends to the polyacrylamide matrix.

We have also developed a manufacturing strategy analogous to replica plating to faithfully copy the polony slide. The polonies of one slide can be sequenced in a highly parallel fashion (see Discussion), and would thus be known for all copies. Duplicate polony slides could then be used for mRNA expression analysis or other applications.

A technique for creating RNA colonies by *in vitro* amplification has been previously described (7). RNA molecules were 'cloned' by performing a Qβ replicase reaction in an agarose gel. However, in this technique, the amplified RNA is not immobilized to the gel matrix, the colonies are grown at a low density and no method was described for fabricating copies of the amplified colonies.

MATERIALS AND METHODS

Primers

The primers used in this experiment are listed below. All primers were obtained from Operon (CA). Sequences common to multiple primers are indicated in bold, italic, underscored or bold and italic. Primer OutF, 5′-**cca cta cgc ctc cgc ttt cct ctc**-3′; Primer OutR, 5′-*ctg ccc cgg gtt cct cat tct ct*-3′; Primer AcrOutF, 5′-Q**cca cta cgc ctc cgc ttt cct ctc**-3′; Primer InF, 5′-<u>ggg cgg aag ctt gaa gga ggt att</u>-3′; Primer InR, 5′-***gcc cgg tct cga gcg tct gtt ta***-3′; Primer AcrInF, 5′-Q<u>ggg cgg aag ctt gaa gga ggt att</u>-3′; Primer PucF, 5′-<u>ggg cgg aag ctt gaa gga ggt att</u> taa gga gaa aat acc gca tca gg-3′; Primer PucR1, 5′-***gcc cgg tct cga gcg tct gtt t***ac acc gat cgc cct tcc caa ca-3′; Primer PucR2, 5′-***gcc cgg tct cga gcg tct gtt t***aa att cac tgg ccg tcg ttt tac aa-3′; Primer PucR3, 5′-***gcc cgg tct cga gcg tct gtt t***ac caa tac gca aac cgc ctc tcc-3′; Primer PucNestF, 5′-**cca cta cgc ctc cgc ttt cct ctc** <u>ggg cgg aag ctt gaa gga ggt att</u>-3′; and Primer PucNestR, 5′-*ctg ccc cgg gtt cct cat tct c***tg ccc ggt ctc gag cgt ctg ttt a**-3′.

The primers AcrOutF and AcrInF have an Acrydite modifi-cation (Mosaic Technologies) at their 5′ ends (designated by

*To whom correspondence should be addressed at: 514 Alpert Building, 200 Longwood Avenue, Boston, MA 02115, USA. Tel: +1 617 432 7562; Fax: +1 617 432 7266; Email: church@salt2.med.harvard.edu

the character Q in the sequences listed above). Acrydite is a phosphoramidite that contains an ethylene group capable of free-radical copolymerization with acrylamide; these primers will polymerize directly into the acrylamide gel as it solidifies (6).

Design of amplification cassettes

The cassette CP-234 was created as follows. The plasmid pUC19 was amplified in a PCR reaction. Fifty microliters of PCR mixture [10 mM Tris–HCl pH 8.3, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM primer PucF, 0.5 µM primer PucR2, 2 ng pUC19 plasmid, 2 U Taq (Sigma)] was cycled in an MJ Research PTC-100 thermocycler as follows: denaturation (1 min at 94°C), 5 cycles (10 s at 94°C, 10 s at 55°C, 1 min at 72°C), 20 cycles (10 s at 94°C, 1 min at 68°C) and extension (3 min at 72°C). The PCR product was purified using Qiaquick PCR purification columns (Qiagen) and resuspended in dH_2O.

To determine the relationship between the length of the amplification cassette and the resulting polony diameter, two more amplification cassettes were created: a 120 bp cassette, CP-120 and a 514 bp cassette, CP-514. These cassettes were created as above, except the reverse primers PucR1 and PucR3 were used instead of PucR2 in the first PCR mixture.

For the replica plating experiments we used cassette CP-281. This 281 bp cassette is identical to CP-234, but it is flanked by two additional primer sites. These sites allowed us to perform nested solid-phase PCR to make duplicate polony slides without contamination from primer–dimer molecules. This cassette was created using the PCR mix [10 ng CP-234, 10 mM Tris–HCl pH 8.3, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM primer PucNestF, 0.5 µM primer PucNestR, 2 U Taq (Sigma)] and cycling it as follows: denaturation (1 min at 94°C), 5 cycles (10 s at 94°C, 10 s at 55°C, 1 min at 72°C), 22 cycles (10 s at 94°C, 1 min at 68°C) and extension (3 min at 72°C). The PCR product was purified using Qiaquick PCR purification columns (Qiagen) and resuspended in dH_2O.

Creating polony slides

To create a polony slide, template DNA was amplified by PCR in a polyacrylamide gel poured on a glass microscope slide. Dilute amounts of template CP-234 (0–360 molecules, quantified by ethidium bromide staining and gel electrophoresis) were added to the solid-phase PCR mix [10 mM Tris–HCl pH 8.3, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 200 µM dNTPs, 10 U JumpStart Taq (Sigma), 6% Acrylamide, 0.32% Bis-Acrylamide, 1 µM primer AcrInF, 1 µM primer InR]. Two 65 µl frame-seal chambers (MJ Research) were attached to a glass microscope slide that had been treated with bind-silane (Pharmacia). 2.5 µl of 5% ammonium persulfate and 2.5 µl of 5% temed were added to 150 µl of the solid-phase PCR mixture. Sixty-five microliters of this solution was added to each chamber. The chambers were then immediately covered with No. 2 (18 mm × 18 mm) coverslips (Fisher) and the gel was allowed to polymerize for 10–15 min.

The slide was then cycled using a PTC-200 thermal cycler (MJ Research) adapted for glass slides (16/16 twin tower block). The following program was used: denaturation (2 min at 94°C), 40 cycles (30 s at 93°C, 45 s at 62°C, 45 s at 72°C) and extension (2 min at 72°C). The coverslips were removed

and the gels were stained in SYBR green I (diluted 5000-fold in TE, pH 8.0) and imaged on a Storm phosphorimager (Molecular Dynamics) or a confocal microscope (Leica).

Determining the relationship between polony diameter, template length and acrylamide concentration

Slides were poured in the manner described above. The ratio of bis-acrylamide to acrylamide was 1:19 for all slides poured. After the slides were cycled, the coverslips were removed and the gels were stained as above. The gels were imaged using the Storm phosphorimager. Any gels with polonies <300 µm in diameter were imaged on the confocal microscope. Care was taken to image only the polonies that could be completely resolved from other polonies. These images were captured and the intensity values saved as a text file. The data was smoothed using a 17-point averaging algorithm and the full width at half maximum of each polony was recorded as its diameter.

Duplicating polony slides

To replica plate polony slides, the 'original' slide is prepared in a slightly different fashion than as described above. The original is a sandwich of two layers of acrylamide, the transfer layer and the readout layer. To create the transfer layer, template DNA is added to a solid-phase PCR mix [10 mM Tris–HCl pH 8.3, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 200 µM dNTPs, 10 U JumpStart Taq (Sigma), 6% Acrylamide, 0.32% Bis-Acrylamide, 1 µM primer AcrOutF, 1 µM primer OutR]. Ten microliters of this solution is then pipetted onto a clean coverslip (18 mm × 18 mm) and the coverslip is picked up by a bind-silane treated slide. The slide is placed in an argon atmosphere to promote polymerization of the acrylamide. The coverslip is then removed leaving a gel ~32 µm in thickness. To pour the readout layer, a fresh solid-phase PCR mix is made; however, no template is added to this mixture. A frame seal chamber is then placed over the transfer layer and, using a bind-silane treated glass coverslip, the readout layer (250 µm) is poured over the 32 µm transfer layer. The slide is then thermal cycled as described above.

When the coverslip is carefully removed from the top of the frame seal chamber, the readout layer will stick to the coverslip, while the transfer layer will be left on the slide. The readout layer can then be stained with SYBR green I and imaged. The transfer layer is then used to make duplicates. To do so, the slide is washed 2× in 10 mM Tris–HCl, 2× in 500 mM KCl, 2× in 10 mM Tris–HCl, 100 mM KCl and 2× in dH_2O. The duplicate gel is then made by placing a frame seal chamber (15 mm × 15 mm) over the transfer layer and pipetting 65 µl of the duplicate solid-phase PCR mix [10 mM Tris–HCl pH 8.3, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM primer AcrInF, 0.5 µM primer InR, 10 U JumpStart Taq (Sigma), 6% Acrylamide, 0.32% Bis-Acrylamide] onto the transfer layer. The duplicate slide is then cycled as follows: denaturation (2 min at 94°C), 25 cycles (30 s at 93°C, 45 s at 62°C, 45 s at 72°C) and extension (2 min at 72°C). Because the coverslip used to pour the duplicate gel was not treated with bind-silane, the gel stuck to the transfer layer when the coverslip was removed; therefore when the duplicate was stained and imaged, the polony pattern was rotated 180° from that of the readout layer.
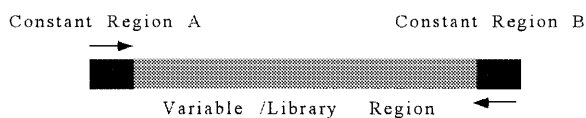
Figure 1. DNA amplification cassette. DNA amplification cassettes contain a variable region flanked by two constant regions. The constant regions contain primer binding sites to allow amplification by PCR. An amplification cassette can be 80–10 000 bp in length.

RESULTS

Creating polony slides

Our goal was to amplify polonies on a glass microscope slide by performing solid-phase PCR (8) in an acrylamide gel. The general design of the template DNA cassettes used to create the polony slide is shown in Figure 1. For most applications, the variable region of each cassette molecule will contain a different DNA fragment. This complex library will contain sequences derived from the genome or cDNA of the organism of interest flanked by constant regions that allow PCR amplification (9). However, to demonstrate and optimize the *in vitro* cloning of DNA, only one species of DNA was used in the solid-phase PCR: the cassette CP-234, a 234 bp template derived from the plasmid pUC19. We included very dilute amounts of the template DNA CP-234 into a PCR mix that contained 6% acrylamide and 0.3% bis-acrylamide. This mix was then used to pour a thin (250 μm) acrylamide gel on top of a glass microscope slide. One of the primers included in the mix contains an Acrydite group at its 5′ end (6) so that it was immobilized in the acrylamide matrix when the gel polymerized. Solid-phase PCR (so named because one of the primers is immobilized to a solid support) was performed by thermal cycling of the slide. The gels went through 40 cycles of denaturation, annealing and extension, and were stained using SYBR Green I.

Upon imaging, green fluorescent spheres were seen in the gels that had been poured with template DNA (Fig. 2A). These spheres were not seen in the control slide lacking DNA template. The spheres were uniform in shape and ~300 μm in diameter, with little variation in size. The number of fluorescent spheres shows a linear dependence on the number of template molecules added (Fig. 2B).

We hypothesized that the fluorescent spheres were polonies that were amplified from a single molecule of the template cassette CP-234. To test this hypothesis, polonies were picked using a toothpick; the toothpick was then dipped into a tube containing a PCR mixture and the mix was thermal cycled. As a negative control, regions of the gel that did not contain polonies were also stabbed with a toothpick. The reactions were then run out on an agarose gel. The results are shown in Figure 2C. The picked polonies clearly show products at 234 bp as expected, while the regions of the gel that contained no polonies yielded no product.

In some experiments, a few larger fluorescent spheres (1–2 mm in diameter) were observed. Because these spheres were also observed on slides that were poured without template DNA, we suspected that these polonies were due to primer–primer mispriming (primer dimer). This was confirmed by repeating the polony picking experiment described above on the putative
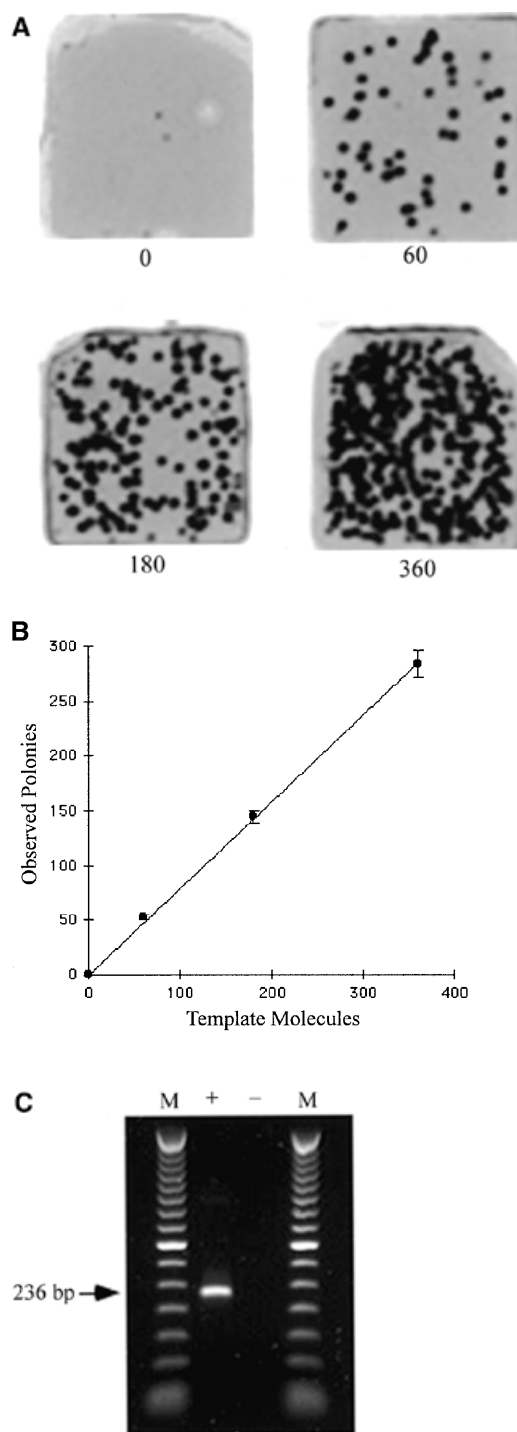

Figure 2. Solid-phase PCR. (**A**) The number of template DNA molecules included in each reaction are listed below each gel image. The polonies are stained with SYBR Green I. (**B**) Plot showing the number of polonies detected versus the number of template DNA molecules included in each reaction. (**C**) Agarose gel (2%) of PCR products from polony picking experiment.

primer–dimer polony (data not shown). We found primer dimer polonies can be reduced or eliminated by raising the annealing temperature of the PCR and/or by careful primer design.
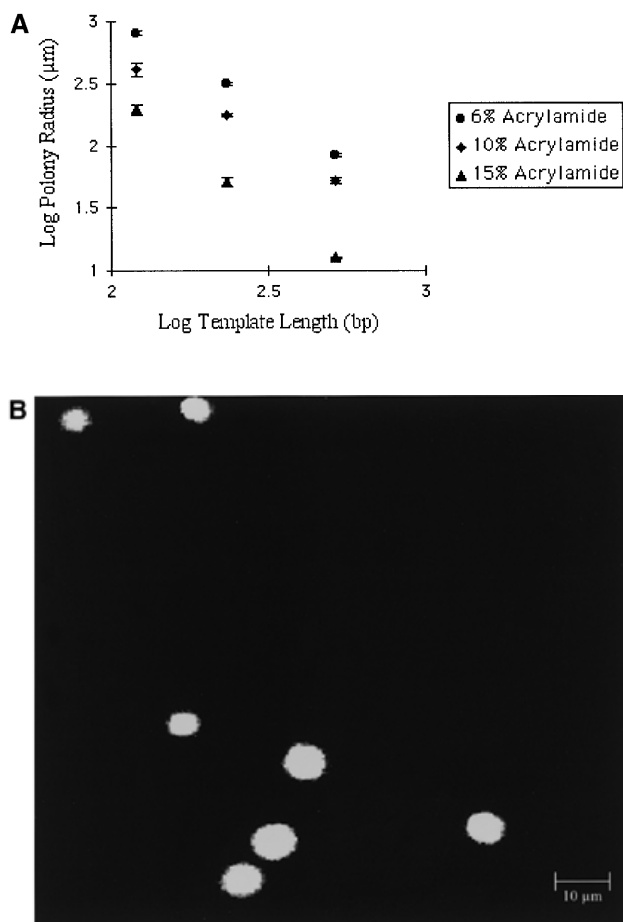
A

Figure 4. Replica plating polony slides. (**A**) The original polony slide. (**B**) The duplicate slide. The image of the duplicate was rotated around its vertical axis to allow comparison with the original.

Figure 3. Relationship between polony radius and length of template. (**A**) Log–log plot showing the relationship between polony radius and template length. (**B**) Confocal image of polonies with an average radius of 6 μM.

Duplicating polony slides

For some applications it would be desirable to make exact copies of a polony slide. Inspired by microbiologists who make replicas of bacterial colonies on agar plates, we developed a replica plating protocol to fabricate copies of our polonies. In this protocol, a polony slide, the 'original,' was created by pouring a thin 32 μm gel containing template DNA (the template layer) on a bind-silane treated glass microscope slide, and then pouring a thicker gel (250 μm) over it. When the original is thermal cycled, the DNA in the thin layer produces polonies that span the interface between the two gels.

When the coverslip was carefully removed from the microscope slide, the thick gel remained intact and attached to the coverslip. This gel was stained with SYBR Green I and saved for comparison with the duplicate. Because the surface of the slide was treated with bind-silane before the original was poured, the 32 μm layer of acrylamide (the template layer) remained bound to the surface of the slide. The slide was washed and a new gel, the 'duplicate', was poured on this glass slide. The duplicate was then thermal cycled and stained.

Figure 4 shows the imaged original and duplicate polony slide. The duplicate slide exhibits a polony pattern that is identical to that of the original. The polonies on the duplicate tend to be slightly larger than those on the original due to diffusion in the duplicate solid-phase PCR reaction.

DISCUSSION

In this report we have described a method for fabricating polony slides containing immobilized DNA by performing solid-phase PCR in an acrylamide gel. We investigated the relationship between the length of the template included in the solid phase PCR reactions, the acrylamide concentration of the gel poured and the size of the resulting PCR colonies, or polonies. In addition, we have described a technique for copying a polony slide once it is created.

Our results provide three pieces of evidence that the stained polonies seen in Figure 2A are due to the amplification of single template molecules. First, the number of polonies obtained in each reaction is linearly dependent on the amount of template included. As seen in Figure 2B, 80% of the template molecules added to each reaction yielded polonies. There are a number of possible explanations as to why 100% efficiency was not obtained: template molecules may have been damaged by the free radicals generated during the acrylamide polymerization, template may have been lost due to abstraction

Relationship between template length, acrylamide concentration and polony radius

To obtain slides with as many polonies as possible, it is necessary to minimize the size of each polony. To determine the parameters that influence polony size, solid-phase PCR reactions were performed using template cassettes of different lengths; acrylamide concentration was also varied. The results are shown in Figure 3.

Polony radius decreases as template length increases and as the acrylamide percentage increases. Using the 514 bp template, CP-514, and an acrylamide concentration of 15%, the polonies produced were very small (average radius of 12.5 μm) and of uniform size (standard deviation of 0.29 μm).

These results showed that polony radius was very sensitive to length of the template. In order to further minimize polony size, we created a template cassette that was 1009 bp long and performed a solid-phase PCR in 15% acrylamide. The resulting polonies had radii of ~6 μm (Fig. 3B). At this size, we estimate that 5 million distinguishable polonies can be poured on a single slide (>13.5 million will be poured on the slide but 63% of these will overlap one another).
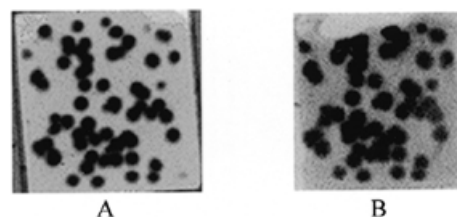
by tube or pipette tip walls, or the amount of template may have been underestimated when quantified by ethidium bromide staining. The second piece of evidence was obtained by a polony-picking experiment. Polonies that were picked and amplified by PCR produced products of the expected length. The third piece of evidence is the strong dependence of polony size on length of the template (Fig. 3), adding further weight to the argument that each polony has grown from a single template molecule.

Reducing polony size is crucial to achieving a high number of clones on each slide, because the number of polonies per slide goes up with the inverse square of the polony size. We systematically varied the length of the template DNA and acrylamide concentration in our solid-phase PCR reactions to reduce polony radius. By using 1009 bp template molecules and 15% acrylamide we obtained polonies that were 6 μm in radius. We believe that the polony radius could be further reduced by increasing the length of the template DNA, by using fewer cycles of PCR or by immobilizing both primers.

The experiments that investigated the relationship between polony radius, acrylamide concentration and template length revealed an interesting phenomenon: small polonies displayed less variation in size than large polonies (Fig. 3A).

To address this question, we developed a simulation for polony growth. This model assumes that at each cycle in the PCR reaction, every DNA molecule will move in a stochastic fashion (due to thermal energy) and then give rise to a complementary strand. The probability that a given molecule will give rise to a complementary strand is dependent on the number of unextended primers and the number of complementary strands in the immediate vicinity of the DNA. We ran this model using a number of different probability distribution functions for DNA motion (all runs assumed that the DNA does not travel too far in relation to the average distance between immobilized primers); in all cases the results were qualitatively similar. This model predicts that template amplification in each polony is exponential during the early amplification cycles. As the polony grows, it will reach a certain radius, the critical radius, after which the amplification proceeds at a polynomial rate. The critical radius is dependent on the diffusion coefficient of the template molecule and the probability that a given DNA molecule is replicated after one cycle of the solid-phase PCR. This phenomenon has a simple physical explanation: one of the primers in the reaction is immobilized; therefore, for a polony to achieve exponential amplification, one strand of each full-length DNA product in the polony must diffuse and anneal to an immobilized primer at each round of amplification. During the early rounds, most of the immobilized primers in the vicinity of a template have not yet been extended so the total number of DNA molecules in a polony increases exponentially with the cycle number. However, in later rounds, the DNA at the center of the polony cannot diffuse far enough to find immobilized primer that has not yet been extended. So, only the DNA near the circumference of the polony can continue to amplify. Therefore, the number of new DNA molecules generated with each cycle increases as the square of the cycle number, so that the total number of DNA molecules in the polony increases with the cube of the cycle number.

The predictions of the polony growth model help to explain why the longer template molecules show less variance in size. When the long DNA template, CP-514, was amplified to form polonies, the polonies reached their critical radii and then grew very slowly for the rest of the reaction. Therefore, all of the polonies tended to be the same size. When the short DNA template, CP-120, was used, the polonies never reached their critical radii, so that some polonies were bigger or smaller than others due to the stochastic nature of PCR.

The replica plating protocol we used enabled us to replicate the polonies on a slide. The PCR mix used to pour the duplicate gel contained primers whose annealing sites were nested inside the annealing sites of the primers used to create the original gel. The nested primers have a higher annealing temperature than the outer primers used to create the original; therefore, when the duplicate layer is poured and the slide is amplified, there will be no extension of the immobilized outer primer in the transfer layer. This means that the polonies in the transfer layer will not increase in radius during the duplicate amplification, so that the transfer layer can be reused to produce a second (or third etc.) duplicate that has no significant difference in average polony radius from the first duplicate. Polonies on duplicate gels tend to be ~15% larger than the polonies on the original due to diffusion of the amplified DNA during amplification.

Applications of polony slides will capitalize on one or more of their four main properties: cloning, immobilization, gel imbedding and *in situ* amplification. Many recombinant DNA cloning procedures typically done at low density on Petri plates can now be done on chips. As many as 5 million clones per slide could be sequenced in parallel using a sequencing-by-synthesis method such as pyrosequencing, which is known to accurately sequence 40 bp of a PCR product (10). This is usually adequate for gene identification or minisequencing (11). A new sequencing-by-synthesis method, solid-phase fluorescent *in situ* sequencing extension quantitation (FISSEQ), would appear particularly suitable (Mitra,R.D., Steffen,M. and Church,G.M., manuscript in preparation). In this technique, the DNA is extended by adding a single type of fluorescently-labeled nucleotide triphosphate to the reaction, washing away unin-corporated nucleotide, detecting incorporation of the nucleo-tide by measuring fluorescence, and repeating the cycle until synchrony is lost. At each cycle, the fluorescence from previous cycles is bleached or digitally subtracted, allowing one to deduce the sequence of each polony iteratively. Because the signal registering successful dNTP incorporation is an immobilized fluorophore, rather than a rapidly diffusing pyro-phosphate, one can uncouple base addition from scanning and can use off-the-shelf microarray scanners.

The ability to sequence polonies *in situ* would enable the rapid resequencing or possibly *de novo* sequencing of small genomes. Polony technology could also be used to measure whole genome RNA or DNA expression. For example, a cDNA library derived from the isolated RNA of interest could be used as template in a polony reaction. FISSEQ could be performed to determine enough sequence information to assign a unique expression tag to each polony. Then one could simply count the relative number of polonies of each tag sequence, and thereby determine the gene's relative expression level. Alternately, one could make duplicates of the sequenced polony slide, hybridize fluorescently labeled mRNA or cRNA preparations, and quantify the amount of fluorescence at each polony. Hybridization in this type of gel-based format provides a 1000-fold increase in DNA layer thickness when compared to DNA attached to a

surface, increasing signal per unit area (12). The high efficiency of polony amplification may allow one to measure whole genome mRNA expression from a single cell. Another advantage of this type of gel format is that it is compatible with miniature gel electrophoretic methods (6,13).

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