genes specifically in the heart without conventional gene therapy approaches.

Advances in fundamental cardiovascular stem cell biology coupled with insights from cardiogenesis and heart regeneration in model organisms are beginning to uncover a growing number of molecular targets for structural forms of heart disease that would have been unthinkable just a decade ago. Yet, at the same time, we are also beginning to realize that acting on many of these molecular targets will be highly dependent on another round of scientific discovery to enable the dose-dependent delivery of therapeutic agents to the heart *in vivo* specifically and directly, and to control their spatial and temporal pattern of expression. Increasingly, the clinical future of regenerative cardiology is likely to intersect with the development of new devices and agents for directly manipulating gene expression in novel ways without gene integration into the genome or conventional viral vectors. In short, as with gene therapy itself, the challenge for regenerative cardiology remains location, location, location. Given the ingenuity of cardiovascular device technology, it will be interesting to see whether the pathway to heart regeneration runs through the cath lab.

COMPETING FINANCIAL INTERESTS
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Single-cell sequencing in its prime

Roger S Lasken

A new method for amplifying DNA allows more-uniform sequencing coverage of the genome of a single cell.

Genome sequencing of single cells is opening new avenues of investigation in such areas as haplotype analysis[1], cancer research[2] and genomic variablity[3]. The key technical challenge in this field is to develop a method for whole-genome amplification that recovers a high percentage of the genome with minimal amplification bias. In a recent paper in *Science*, Zong *et al.*[4] present such a method, called multiple annealing and looping-based amplification cycles (MALBAC), and show that it amplifies genomic DNA from single human cells with less bias than previous methods. The approach enhanced the sequencing of both parental chromosomes with even coverage, increasing the accuracy with which heterozygous single-nucleotide variations could be genotyped. Although uncertainties about the MALBAC method remain, it is an important new strategy for reducing bias in whole-genome amplification methods that will advance single-cell genomics.

For more than a decade, the most reliable method for amplifying a whole genome from a single cell has been multiple displacement amplification (MDA)[5,6], developed in my and other laboratories. In this reaction, random primers are extended by a strong strand-displacing enzyme, the phi29 DNA polymerase. As the polymerase extends the growing DNA strands, it displaces downstream strands resulting in a branching form of amplification.

Compared with earlier whole-genome amplification methods that relied on PCR, MDA reduces amplification bias by three to four orders of magnitude and generates far larger amplicons with average length >12 kb[6], thereby yielding more-uniform coverage of the genome. The resulting coverage is sufficient for extensive sequencing of single bacterial cells and has made it possible to analyze the vast numbers of uncultured microbial species[7].

Yet MDA is not completely unbiased. Using it to genotype single-nucleotide variations in diploid human genomes from a single cell has not been successful; amplification bias can result in failure to detect an allele for one of the parental chromosomes, leading to heterozygous loci being miscalled as homozygous. Despite the efforts of many laboratories, no reductions in the amplification bias of MDA have been reported in the 12 years since it was introduced. The field has advanced primarily through statistical strategies that analyze candidate mutations from multiple single cells and bulk DNA extractions[8,9]. For example, by sequencing multiple single cells from the same specimen, it has been possible to identify mutations present in only ~10% of the cells in a tumor.

MALBAC directly addresses the underlying causes of amplification bias by combining features of MDA and PCR (**Fig. 1**). Primers consisting of eight nucleotides of random sequence and a common 27-nt tag are annealed to template DNA and are extended by a polymerase in an isothermal strand-displacement reaction, similar to MDA. Unlike MDA, however, MALBAC uses repeated short cycles of primer extension followed by denaturation at 94 °C. The authors describe this as 'quasilinear' amplification, in which initial priming events are more-evenly distributed over the course of multiple cycles during which the reaction rate is limited. In addition, the ends of the newly made amplicons base pair by means of the 27-nt sequence common to each primer, forming loops that inhibit the 3′ ends from serving as primers. After five cycles of quasilinear amplification, the resulting DNA is amplified by PCR to a level sufficient for sequencing.

Zong *et al.*[4] showed that MALBAC results in less amplification bias than MDA, based on coverage data from Illumina sequencing. The efficiency of detecting both alleles of known single-nucleotide variations was 71% compared to only 10% for MDA.

Detailed studies have not yet been published on the reaction mechanisms of MALBAC or the contribution of the individual steps in reducing amplification bias. For instance, it is not clear what role the looping plays in altering the kinetics or the outcome of the reaction. It will be important to evaluate how much of the reduction in bias can be attributed to the cycling of initial priming or to the loop structure of the amplicons. Presumably, MDA could also benefit from early cycling as a means to produce more-even priming, a possibility that remains to be tested.

Additional studies will be required to investigate MALBAC's performance and its use in particular applications. Although it had a low false-negative rate for detecting heterozygous loci, the false-positive rate was high, primarily due to misincorporation of nucleotides by the DNA polymerases. These alterations will be incorrectly identified as new mutations. Zong *et al.*[4] sequenced DNA from single cells amplified with either MALBAC or MDA and found that the false-positive rate for genotyping single-nucleotide variants with MALBAC was about 40-fold higher than for MDA. The likely explanation is that MDA uses the high-fidelity phi29 DNA polymerase whereas MALBAC uses two relatively error-prone DNA polymerases, the large fragment of *Bacillus stearothermophilus* (*Bst*) DNA polymerase for

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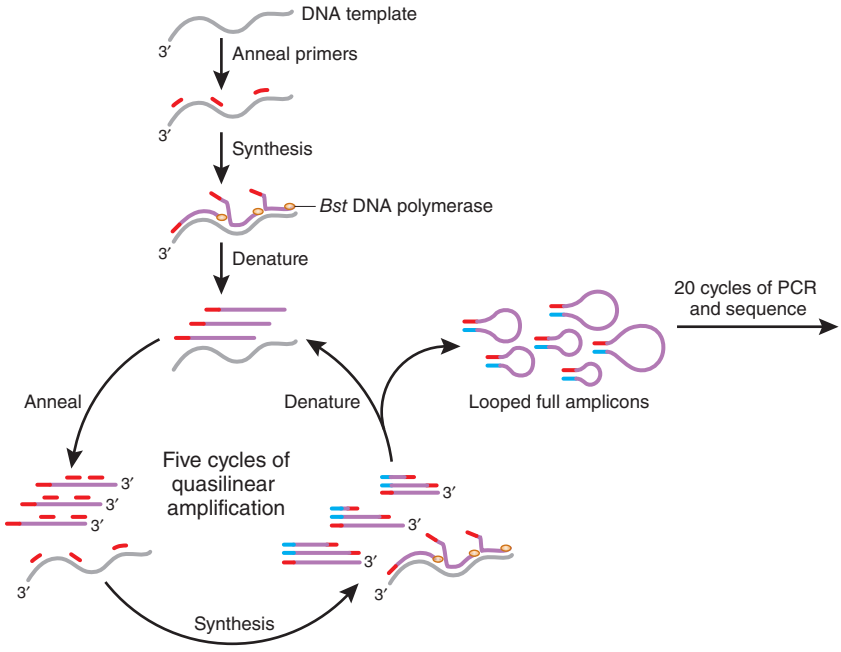

Katie Vicari

Figure 1 Simplified diagram of the MALBAC reaction. MALBAC primers (red) having a 27-nt common sequence followed by eight random nucleotides are annealed to the genomic DNA template. Strand-displacement synthesis generates partial amplicons, which are subsequently denatured from the template at 94 °C. Priming to new positions on the genomic DNA template generates more partial amplicons, which increases coverage of the genome with a resulting reduction in amplification bias. Priming and extension on the partial amplicons yield complete amplicons having the MALBAC primer sequence at the 5′ end (red) and its complementary sequence at the 3′ end (blue). Denaturation at 94 °C regenerates the original template and a now larger and more diverse pool of partial amplicons. Full amplicons form loops, which may be resistant to subsequent amplification and hybridization. Full amplicons are generated for five cycles and then exponentially amplified by 20 cycles of PCR using primers complementary to the common region of the MALBAC primers. *Bst, Bacillus stearothermophilus.*

isothermal strand displacement and Taq DNA polymerase for PCR.

To overcome the false-positive errors, Zong *et al.*[4] sequenced several single cells from a highly homogeneous cell culture. Briefly, a single cell was isolated from the culture and propagated through three cell divisions to obtain highly clonal kindred cells. DNA from three of these cells was amplified by MALBAC and analyzed by high-throughput sequencing. Sequences of single cells were compared with the predominant sequence of a bulk DNA extraction from the culture, allowing the identification of rare cell-specific alleles. It remains to be seen how well such an analysis can be carried out with more-complex cell populations such as tumor cells. Variants found in all three cells were deemed validated, whereas variants found in only one or two cells were false positives generated by MALBAC. Thus, it appears that statistical strategies using multiple single cells will still be required for many studies using MALBAC. There may be ways to reduce the false-positive error rate by adapting the protocol to use a high-fidelity enzyme, such as phi29 DNA polymerase, or a thermostable DNA polymerase with strong proofreading activity. The 20 cycles of PCR that are required might also be optimized to reduce error rates.

A second limitation of MALBAC involves regions of the genome that are reproducibly underrepresented. In the experiments of Zong *et al.*[4], about 20–30% of known single-nucleotide variants could not be accurately genotyped even when multiple cells were pooled in a single MALBAC reaction. Sequences in certain contexts can be difficult for DNA polymerases to copy. For instance, regions of secondary structure in the DNA template might be poorly replicated during the strand displacement or PCR steps. A difficult template sequence for either of the DNA polymerases used would result in poor recovery in the final MALBAC product. A published analysis of the sequences in the human genome that are consistently underrepresented would help users of MALBAC better design their research strategies.

As with PCR and MDA, there will be much to learn about MALBAC reaction mechanisms and the DNA products that are generated. For example, chimeric DNA rearrangements that occur in MDA[10] have been detected in MALBAC. However, their rate of formation has not yet been reported. Although uncertainties remain, MALBAC is a notable advance because it highlights the importance of the initial priming events in whole-genome amplification. The data demonstrate that bias can be reduced by controlling this crucial phase of the reaction and that genotyping of a diploid cell at the single-nucleotide level is feasible. A period of rapid improvement and testing of applications can be anticipated.

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