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The applications of single-cell genomics

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We all start out as a single totipotent cell that is programmed to produce a multicellular organism. How do individual cells make those complex developmental switches? How do single cells within a tissue or organ differ, how do they coordinate their actions or go astray in a disease process? These are long-standing and fundamental questions in biology that are now becoming tractable because of advances in microfluidics, DNA amplification and DNA sequencing. Methods for studying single-cell transcriptomes (or at least the polyadenylated mRNA fraction of it) are by far the furthest ahead and reveal remarkable heterogeneity between morphologically identical cells. The analysis of genomic DNA variation is not far behind. The other 'omics' of single cells pose greater technological obstacles, but they are progressing and promise to yield highly integrated large data sets in the near future.

INTRODUCTION

Biologists have long been fascinated by the molecular differences between individual single cells within tissues and organ systems. What is different between adjacent cells, how do those neighbors influence each other, how do they influence the structure and function of the organ and organism? How do they differ at the genetic, epigenetic and gene expression level? How do single cells obtain, retain or modify specific developmental or disease fates? These questions remain largely unanswered. They have obvious relevance to processes such as commitment and differentiation in early embryogenesis, where individual cells are rapidly acquiring different fates. In disease states, tumors, for example, exhibit cellular heterogeneity, which, as a given single tumor progresses and evolves, affects the malignant phenotype. However, it is still unclear which individual changes or combination of changes occur at the single-cell level to then drive the evolution of an aggressive versus a more benign tumor? Recently, these questions have become system-wide. It has become clear that remarkable cell-to-cell heterogeneity exists between the transcriptomes of single cells from apparently homogeneous organ cell types [e.g. (1)], although the exact range of this variation across multiple tissues and organ systems is still unclear. What ties all of these interesting questions and phenomena together is the technological need for methods to accurately explore the 'omics' of single cells; from genetic/epigenetic variation within genomic DNA to the complete transcriptome, the proteome and the metabalome. Ideally, one would wish to extract all of these data sets simultaneously from each cell. At present, extracting any one of them is still complex and challenging.

The technological hurdles are formidable. An average human cell measures in the tens of microns and has a cytoplasmic volume of ~1 pl. Within this reside ~6 pg of genomic DNA, ~20 pg of total RNA, ~10^9 proteins, ~10^{10} lipid molecules and 10 pg of carbohydrates (2) (to name just some major constituents). Accurately measuring all of that information content from within such a small object is difficult. The first, and arguably largest, obstacle is physically capturing a single cell. This has been variously achieved by micropipetting (3) (a technique probably best left to the experts), FACS sorting (4) (a long-established, but somewhat time-consuming, technique) and microfluidics (5–8). The latter methods have been commercialized by several companies, are being increasingly adopted and hold great promise. They result in the sorting of individual cells and they also place them into devices where the contents of multiple single cells can be simultaneously, separately extracted and analyzed in nanoliter volumes. Thus, the various biochemical steps of macromolecular extraction can be rapidly conducted by microfluidics on each partitioned tiny sample to minimize losses and yield the starting substrates for further analysis. For RNA analysis, it is clear that multiplexed QRT-PCR for ~96 individual transcripts from single cells has been reduced to practice in these systems (6,7). Assaying ~96 genes falls a bit short of the 'genomics' hurdle, but it is an encouraging step forward and can nevertheless provide immediate and interesting insights. For example, even this relatively small number of QRT-PCR assays (7), when conducted on many

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microfluidics-separated single cells (1440), identify important, heritable differences in gene function and expression that are masked by other methods that only measure 'average' gene expression values from mixed cell populations. Microfluidics have become key enabling components in all of single-cell genomics, including the nascent fields of single-cell proteomics and metabalomics (8–10).

SINGLE-CELL TRANSCRIPTOMES

Time is of the essence in transcriptome studies, lest the cell change its gene expression profile while being dissociated, sorted or otherwise manipulated. RNA polymerase II has an average elongation rate of several kilobases per minute, so extensive times between tissue collection and single-cell separations can influence such experiments. Once a single cell is (rapidly) isolated, the next technical challenge is to extract the contents one wishes to analyze. When dealing with mRNAs, this usually involves isolating total RNA, then selectively isolating and amplifying polyadenylated (polyA+) mRNAs. The vast majority of single-cell transcriptome studies have focused on the profile of these mRNAs. The selected RNAs are then converted into cDNAs which are much more stable and can be (relatively) easily sequenced. Although many different flavors of reverse transcriptase (RT) have been developed, most still suffer from low processivity and a propensity to template-switching (more on this below). These effects can be tolerated when dealing with large RNA samples, but when seeking to analyze a single-cell transcriptome of ~300 000 polyA+ molecules, the aim is 100% capture and 100% conversion of every mRNA. The technical problems do not end there. Every single-cell RNA or DNA protocol involves some form of amplification, with all of the possible problems in skewed sequence representation and abundance that these can involve. Close attention must be paid to devising and including many internal controls. Until the day that single-molecule DNA/RNA sequencing becomes truly feasible, it is difficult to see how amplification methods can be avoided. For Next-Generation deep sequencing analyses such as RNA-seq, one must also add specific linkers to the amplification products. This introduces another level of variability into the process. The availability of new one-step fragmentation and linker addition (tagmentation) methods [such as the Tn5 transposase-based Nextera system from Illumina (11)] may help in increasing the efficiency of these steps.

Surani and co-workers (3,12,13) have been among the foremost practitioners in developing and using micro-cDNA methods, particularly in applying them to important questions in early mouse embryogenesis and the establishment of embryonic stem cells. However, even here the basic protocols employed for cDNA synthesis have varied little in decades. The core recent innovations have been the ability to capture a single cell in a tiny volume within a micropipette, the development of relatively mild extraction procedures that allow for rapid conversion to cDNA and the use of custom nested primer sets to improve the fidelity of the PCR amplification process (3). These clearly work well in expert hands.

More recently, two papers have described the application of so-called SMART-Seq to single-cell transcriptome analysis (1,14). This system (shown diagrammatically in Fig. 1) takes advantage of the intrinsic terminal transferase (TdT) and template-switching activities of MMLV-RT. cDNAs are oligodT-primed as usual, but at the end of the first-strand synthesis, the TdT activity of the RT adds a few non-templated nucleotides (shown as Cs in Fig. 1). This allows template-switching to an oligonucleotide linker containing the complementary residues. This is marketed as a kit and was originally described and commercialized over a decade ago (15), but the recent publications demonstrate that it works in conjunction with RNA-seq analysis and can reveal significant single-cell transcript and splice isoform heterogeneity (1). We (16–19), and others (20), have modified this system over the past decade to incorporate some features that expand its utility. Two of these are shown in Figure 1. The first is the use of oligo-dT-linked beads as a priming source, which allows for reuse of the original sample several times over. The second is the use of linkers at each end of the cDNA that incorporate T3 and T7 promoters. These allow for the use of either exponential PCR amplification methods or linear *in vitro* transcription methods (or a combination of both) to generate larger quantities of the transcriptome sequences. In the case of the two recent RNA-seq studies (1,14), only PCR was employed (~18 cycles) for single-cell analysis. In common with most other single-cell transcriptome studies (1,3,12–14), the number of reproducibly detected transcripts was lower (~6000–8000) than is found for larger scale RNA-seq (~15 000). This probably reflects the technical limitations (mentioned above) in efficiently capturing and converting all of the low-abundance mRNAs from a single cell, but it may also reflect stochastic or 'noisy' variation in gene expression between individual single cells (21). Despite these limitations, the results of these studies are encouraging and suggest that, even without microfluidics, single-cell RNA-seq is reasonably feasible and reproducible for more abundant poly A+ mRNAs.

The non-polyadenylated mRNA population and the diverse populations of regulatory non-coding RNAs [nc-RNAs, together estimated at ~20% of the polyA+ levels (22)] have been largely unexplored at the single-cell level. Surani and co-workers have (23) explored the miRNA transcriptome of single cells by constructing specific hairpin amplification adapters for a targeted subset of the miRNA transcriptome. However, complete single-cell miRNA transcriptomes remain a considerable challenge. These RNAs are very short and standard protocols require size selection steps for accurate sequence analysis. Nevertheless, it may prove feasible to dispense with these steps for single-cell miRNA analysis. Likewise, the other regulatory non-coding RNAs have not yet been targeted from single cells. The established method for isolating these molecules from large populations of cells is to deplete the vast excess of rRNAs from the total RNA mixture (e.g. 24). One can envision that some form of 'sponge' for these abundant RNA species could feasibly be coupled with microfluidics to deplete them from single-cell transcriptomes.

GENOMIC DNA SEQUENCING FROM SINGLE CELLS

Numerous studies over the past decade have made use of whole-genome amplification (WGA) methods to obtain sufficient DNA for sequence analysis. Predominant among these applications

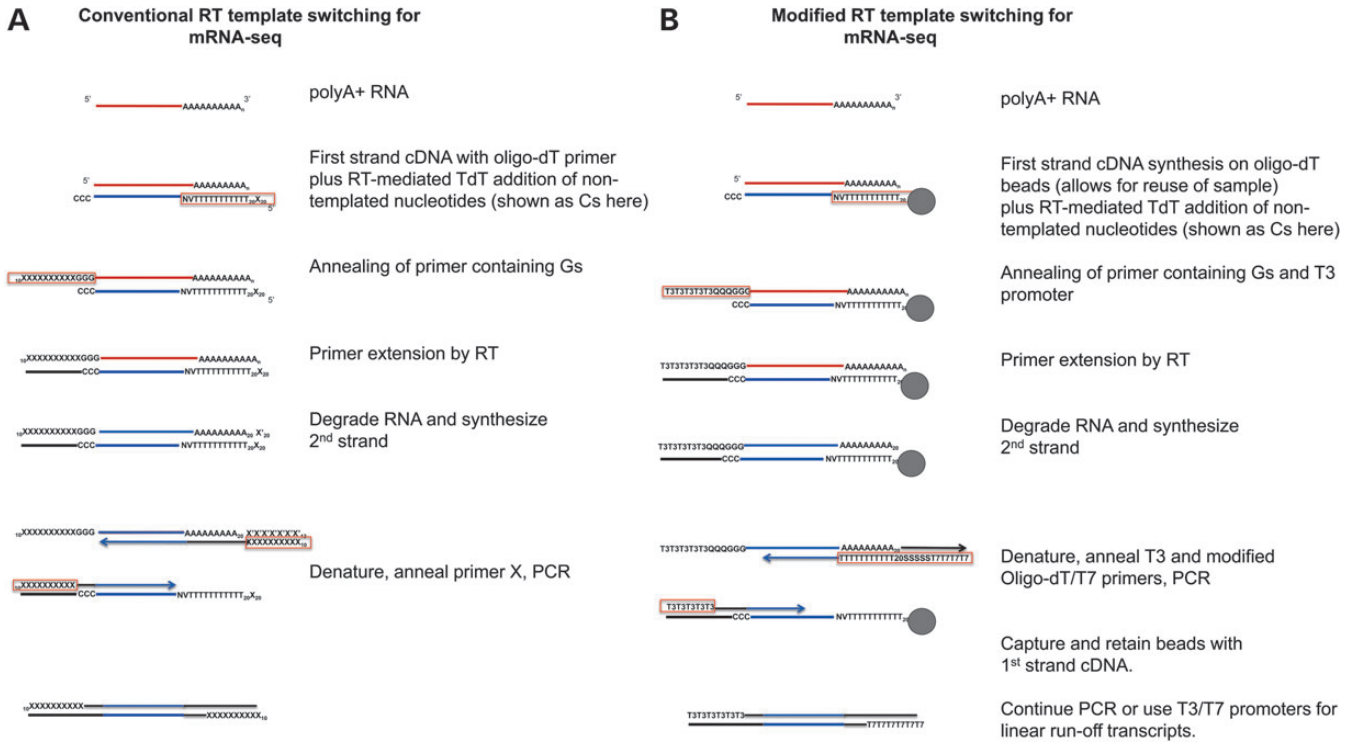

Figure 1. cDNA template-switching and modifications for single-cell mRNA transcriptomes. (**A**, left) The conventional 'Smart-Seq' system as applied in references (1) and (15). (**B**) Modifications from our group that enhance these techniques (16–19). The first is the use of oligo-dT-linked beads as a priming source, which allows for reuse of the original sample several times over. The second is the use of linkers at each end of the cDNA that incorporate T3 and T7 promoters. Oligonucleotide linkers are shown in boxes. The cDNA sequence is shown in blue, and the RNA sequence is shown in red. Subscripted letters indicate a longer number of nucleotides. X equals a PCR priming sequence and X? is its complement. For 'Smart-Seq', a common primer is used at both ends of the cDNA construct. The boxed constructs in (B) refer to the T3 and T7 promoter sequences for run-off RNA synthesis and are not to scale. V = T, G or C. N = any nucleotide. Q and S = non-promoter spacer sequences.

has been multiple displacement amplification [MDA (25)], which uses a strong strand displacing DNA polymerase to achieve a branching form of amplification. Although this is an improvement over conventional PCR, it results in amplification biases (26), whether they be in single-nucleotide variant calling or copy number studies (27). Nevertheless, these methods have proved extremely useful in recent single-cell whole-genome analyses and particularly in studying the evolution of tumor phenotypes by low-coverage single-cell sequencing (28). However, a recent paper describes a new WGA method that considerably improves on amplification fidelity (29). This multiple annealing and looping-based amplification cycle (MALBAC) method is shown in Figure 2. It combines some features of linear amplification methods with PCR. The first steps are short cycles of 'quasilinear' amplification to generate amplicons that can then be amplified by PCR. The 'trick' here is that the primers in the initial reaction are designed to share common sequences that form loops and inhibit the repeated (potentially biased) priming from their ends. The net result is an improvement in coverage from ~6% for MDA (28) up to ~90% for MALBAC (29) and an improvement in detecting both alleles of a given SNP from ~10% for MDA to ~70% for MALBAC. This is impressive and should find wide applications in single-cell genomic analyses.

The epigenetic analysis of single genomes is a less encouraging prospect. There are methods for analyzing single genomes with methylation sensitive restriction enzymes (30), but they are far from comprehensive and have not been widely adopted. Chromatin immunoprecipitation of histone marks or methylation marks is difficult on even thousands of cells (31) and is far from being amenable to single-cell analysis. Sequencing of bisulfite modified DNA is the current gold standard method for interrogating the methylome. However, this is a difficult chemistry at the best of times and can result in dramatic DNA losses that cannot be tolerated for single-cell genomes of 6 pg. The amplification of these modified DNAs is also problematical (32). One strategy that has not yet been explored is to couple WGA techniques such as MALBAC with replication of the methylation marks within the genome. This might produce enough templates for the established methods. The maintenance DNA methylase Dnmt1 (33) acts on hemimethylated DNA *in vivo* to potentiate these marks and might be employed to potentiate MeCpG marks during the sequence amplification process *in vitro* (34).

PROTEOMICS AND METABOLOMICS IN SINGLE CELLS

The study of proteins and metabolites [the latter traditionally considered as molecules <1 kDa in molecular mass (2)] is understandably behind that of nucleic acids. There is no amplification methodology for these molecules that is comparable with PCR for RNA (cDNA) and DNA. Nevertheless, these fields have

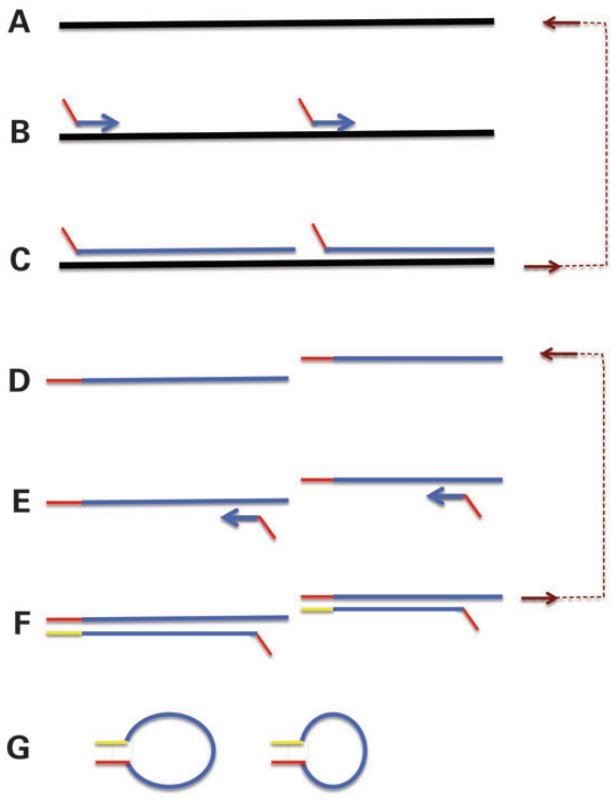

Figure 2. The MALBAC system for WGA. Genomic DNA (shown as a single strand in **A**) is denatured and annealed to a series of random primers (8-mers) that all share a common 27 base tail (shown in red, **B**). Extension of these primers leads to a series of products that are all terminally tagged (in red, **C**). These are then put through another round of denaturation, annealing and extension (**D** and **E**). This results in extension products that have a (red) MALBAC tag at one end and its complement (shown in yellow) at the other end (**F**). These are annealed to form loops and remove them from the amplification reaction (**G**). After five rounds of this 'quasilinear' pre-amplification, the annealed loops are used as the substrates for conventional PCR using the MALBAC tags (the terminal red tags they all share). Adapted from reference (29).

made considerable progress, partly through microfluidics, but also because of interdisciplinary research teams that bring together engineers, physicists, biologists and bioinformaticians. One solution to the proteomics problem has been to engineer a thousand individually fluorescence-tagged proteins by separate gene replacement of each gene in thousands of individual cells (35). The response of each of these individual constructs to a treatment [e.g. a chemotherapeutic agent (35)] can then be measured by high-resolution imaging. This is a technological tour de force, but is very costly in time and labor and requires that only one or a few individual protein products can be measured per cell. Another imaging method that works on a much smaller scale is the sequential detection of a dozen or so proteins by repeated cycles of fluorescence detection on formalin-fixed tissues (36). An alternative strategy is to employ microfluidics/'lab on a chip' methods to capture and interrogate multiple proteins in small volumes (8,9,37–39). These are label-free methods that also allow for cellular fractionations to be conducted on the chip. Protein detection and quantification is by specific antibodies to individual proteins. These methods critically depend on the availability of two antibodies per protein (one for initial capture and the other for quantification). To date these methods have been reduced to practice for a few dozen protein targets and they show great promise, but there is a need for additional and alternative specific capture and detection methods for the huge numbers of protein targets in a single cell.

The diversity of metabolites in a single cell is quite staggering. The current single-cell methodologies in this field again seek to combine established analytical tools (e.g. mass spectroscopy) with microfluidic devices. At present, these emerging technologies can measure a dozen or so, highly abundant, metabolites from a single cell (2,10).

CONCLUSIONS AND FUTURE PROSPECTS

There has been considerable recent progress in analyzing single-cell genomes and mRNA transcriptomes. There remains considerable room for improvement and a continued need for careful internal controls, so we are not quite at the 'off-the-shelf' kit stage of most routine molecular biology techniques. However, partly through innovations in microfluidics and Next Gen sequencing technologies, the primary nucleic acid sequence analysis of single-cell genomic DNAs and polyA+ RNAs are close to being solved technological problems. In the near future, we should expect to see the analysis of ever smaller and sub-partitioned parts of single cells—organelles, single chromosomes, nc-RNAs, full-length transcripts, membrane proteins, to name just a few. This single-cell revolution will require not only the continued development of analytical platforms for reproducible 'plug and play' single-cell genomics, but also bioinformatic tools that can make sense of the imminent data deluge.

Conflict of Interest statement. None declared.

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