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REVIEW ARTICLE

A strategy to identify genomic expression at single-cell level or a small number of cells

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Recent advances in functional genomics allow us to estimate the expression of several thousands of genes in the mammalian genome. Techniques such as microarrays, expressed tag sequencing (EST), serial analysis of gene expression (SAGE), subtractive cloning and differential display (DD), and two-dimensional electrophoresis gel have been extensively used to screen and analyze parallel gene expression. Some pathological processes, for example, tumorigenesis and solid tumour growth, in which the former is derived from a single-cell and the latter has a mixed-cell problem, present new challenges to the limit of these functional genomic techniques. To fully understand the functions of cells in tumorigenesis or in heterogeneous solid tumour masses, it is essential for scientists and physicians to develop a strategy to identify genomic expression profiles for a single-cell or for small numbers of cells. In this article, we review recently developed methods that enable functional genomic analysis at the single-cell or multi-cell level. In addition, the paper will review different techniques of single-cell genomic expression at the level of DNA, mRNA, protein and post-translational modifications.

A major task of functional genomics is to study cell function at the level of mRNA and protein expression. Routine approaches of identification and quantification include DNA microarrays, expressed tag sequencing (EST), serial analysis of gene expression (SAGE), subtractive cloning and differential display (DD) for mRNA, and two-dimensional gel electrophoresis, mass spectrometry and protein microarray based antibody-binding for protein. Traditionally, each approach requires relatively large numbers of cells (Kim, 2002; Steinert et al. 2002).

These traditional methods have been extensively utilized to study parallel gene expression in different cell lines. It is known that cell lines used as models in some fields, such as tumorigenesis, have limitations. For example, after certain tumour cell lines go through several hundred passages, many properties of tumour cells have changed; the genome expression from the cell line does not accurately reflect properties of *in vivo* tumour cells. However, some primary tumour cells from tumour tissue display the intrinsic function and properties of tumour cells. In addition, tumour cell formation and development involves the accumulation of multiple-gene mutations as a tumour grows from a single cell or a very small number of cells (clonality) (Boultwood and Wainscoat, 2001; Wood, 2001). If the single cell or a small number of cells from primary tumour cells can be employed for genome analysis, it can address questions such as how tumour cells form, how tumour tissue develops, and how some agents can block tumour formation and development. Based on the requirement for analyzing tumorigenesis, some strategies have been developed to study functional genomics at the single-cell level.

In order to clearly introduce functional genomic analysis in the single cell, we will briefly review traditional functional genomic methods and then discuss genomic analysis at the unicell level.

TRADITIONAL GENOMIC METHODS

Genome-wide monitoring of levels of DNA, mRNA and protein

Many traditional techniques used to measure gene expression are directly related to the quantitative detection of mRNA and protein among parallel samples. Relating these techniques to the central dogma (Figure 1) can help us to categorize these methods. Briefly, they can be divided into four fields: (1) DNA level such as genomic sequencing and single nucleotide polymorphism (SNP); (2) mRNA level including microarray, expressed tag sequencing (EST), serial analysis of gene expression (SAGE),

subtractive cloning and differential display (DD); (3) protein level, for example, two-dimensional gel electrophoresis, mass spectrometry, and protein arrays based on antibody binding; and (4) post-translational level such as protein-protein interaction *via* the yeast two-hybrid or repressor system (Cordwell et al. 2000; Dean, et al. 2002; Forde and McCutchen-Maloney, 2002; Meehan and Welch, 2003; Shaknovich, 2003).

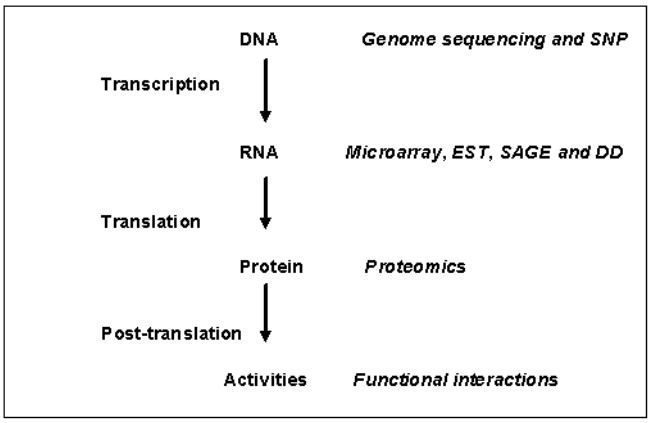

Figure 1. Traditional genomic study.

In general, the extreme stability of genomic DNA allows it to be used to examine single nucleotide polymorphisms (SNP). A large-scale analysis of SNPs can help use to identify genes affecting variant human phenotype, including different diseases and drug responses in some individuals. Although genomic DNA has these advantages, it is not extensively used for gene expression and gene regulation at the cellular level (Lilleberg, 2003).

Functional genomic studies at the mRNA level have demonstrated tremendous development in the past few years, evidenced by the development of spotted-based microarray, library-based EST and SAGE and gel-based differential-display. These mRNA-based methods are critically important for screening and analysis of mRNA regulation and deregulation in whole cell systems. However, such approaches will overlook changes in post-transcriptional regulation, protein expression level, and post-translational modifications (Weigl et al. 2003).

Further, emerging proteomic strategies such as two-dimensional gel electrophoresis and mass spectrometry will provide proteomic information regarding protein expression levels and post-translational modifications. However, protein stability and small concentration of protein available for the study will limit the application of these traditional methods at the single-cell level (Murray et al. 2002).

Material influencing genomic study at the level of DNA, mRNA and protein

Before discussing genomic expression at the single-cell level or at level of a small number of cells, scientists should carefully consider issues of material sources and procurement. In general, human tissue is available from biopsy or surgical specimens. These specimens can be available in 3 different forms: they can be freshly obtained, frozen, or embedded in paraffin. First, and perhaps most important, the quality and intactness of biomaterials including DNA, mRNA and protein need to be addressed here. According to routine specimen procedures involving genomic study, there is little doubt that fresh tissues or culture cells are the best choice to analyze genome expression. Fixed tissues from animals and humans used in gene expression have yielded inconsistent results. Some papers demonstrate variable results with the use of fixed tissue for expression analysis, while others have successfully measured levels of gene expression. Interestingly, some reports demonstrate no difference in either the quality or intactness of RNA extracted from fixed tissue (by formalin and ethanol) and from standard frozen tissue when evaluated by reverse transcription combined with PCR amplification (Morita et al. 1994; Ren et al. 2000).

A second area of importance is the quantitative requirement of DNA, mRNA and protein from biomaterials. Varying levels of DNA, mRNA, and protein yield have resulted in different results due to the use of different models of equipment, the use of different protocols to purify DNA, mRNA and protein and variability in user expertise. Here, I include minimal concentration requirements for some emerging techniques (Table 1). The concentrations of DNA, RNA, protein and the approximate cell numbers are based on some traditional methods. The details of developments regarding sensitivity and resolution of the parallel expression of genome will be discussed in the next section.

FUNCTIONAL GENOMICS AT SINGLE-CELL LEVEL

Specimens of animal and human tissue often contain multiple cell types with different gene expression profiles. Theoretically and practically, potentially important findings in the gene expression profiles in the multiple cell types will be obscured or unclear. Therefore, studies of representative single cells will provide the most precise analysis possible of these subtle gene expression patterns. Here, in order to discuss functional genomics in single cells clearly, the following three fields: (1) single-cell sampling; (2) mRNA and protein isolation or amplification from a single cell; and (3) application of genomic expression of single cells, will be systematically reviewed.

Single-cell sampling

As shown in Table 2, flow-cytometric cell sorting and laser-based microdissection of tissues now provide good methods to isolate single cells for gene expression profiling. In flow cytometry, cells in solution are labeled

Table 1. Traditional functional genomics *.

Methods	Minimum Material	Throughput	Sequencing	Application
Sequencing base				
EST	1-5 ug polyA RNA	High	High	gene expression
SAGE	1-5 ug polyA RNA	High	High	gene expression
Gel base				
DD	10-100 ng polyA RNA	Medium	Low	Parallel gene expression
Colony base				
Subtractive cloning	10-100 ng polyA RNA	Medium	Low	Parallel gene expression
Hybridization base				
Microarray	>1 ug polyA RNA	High	N/A	Parallel gene expression
Protein base #				
Two-D gel electrophoresis	10^6-10^7 cells	High	N/A	Parallel protein expression
Mass spectrometry	10^5-10^6 cells	High	N/A	Parallel protein expression
Functional array				
Repressor system	N/A	N/A	Low	Protein-protein reaction
Yeast- library system	N/A	N/A	Medium	Protein

* Sample concentration: Claudio et al. 1998; Trendelenburg et al. 2002; Bosch et al. 2000; Carulli, et al. 1998; Oppermann et al. 2000.
# Some journals used as protein concentration

with fluorescent signals. These signals can be derived from a specific biomarker such as a tumour antigen attached to an antibody that is labeled with a fluorescent signal or a recombinant DNA construct encoding modified proteins with a fluorescent signal. At present, multi-coloured fluorescence-activated cell sorters can selectively separate and collect homogeneous cells with identical phenotypic features in a collection tube in order to increase sensitivity for gene expression profile in a given cell type (Ormerod, 2000). Although flow-cytometric cell sorting and multi-colour fluorescence-activated cell sorters can isolate and sort homogeneous cells, there are three limitations to using these techniques: (1) some cell types such as neurons are not amenable to separation and sorting by flow-cytometry; (2) internal cell localization of sub-cellular components cannot be well-defined using flow cytometry; and (3) the microenvironment of a cell (such as a good blood supply or a bad blood supply) cannot be evaluated and studied using flow cytometry.

The microdissection technique, in part, avoids these problems. The use of lasers in tissue microdissection was reported as early as 1976 (Meier-Ruge et al. 1976), and it has increasingly been applied in single-cell microdissection in recent years. In contrast to flow-cytometric cell sorting, microdissection allows for both rapid *in vivo* localization and the ability to analyze the cellular microenvironment (Schutze and Lahr, 1998). At present, three microdissection methods have been developed:
(1) laser-assisted mechanical tissue microdissection;
(2) laser pressure catapult microdissection;
(3) laser capture microdissection.

Laser-assisted mechanical tissue microdissection can focus on small target cell areas, reducing the chance of contamination with neighbouring cells (Emmert-Buck et al. 1996). Although the concept of using a laser to dissect out individual cells is quite simple, the technique is laborious. Laser pressure catapult microdissection concentrates on an interesting region with a high-energy cutting laser. First, a low-power laser sets the depth of the tissue section; a pressure wave then separates the targeted tissue from the slide and catapults it into a receptacle. The high precision of the thin-beam laser is sufficient to isolate sub-cellular targets such as chromosomes - a distinct advantage for this technique. The absence of physical contact between the surrounding tissues and the collection apparatus results in a much lower incidence of contamination. In laser capture microdissection, a thin ethylene vinyl acetate film is mounted on the tissue section. After an infrared laser heats and melts a cell of interest, the re-solidified plastic film binds directly to this cell and catches it. Potential problems of laser capture microdissection as shown in Table 2 include damage to the target cell by contact with heat film (Fend et al. 1999). At present, all of these methods are commercially available for laboratory and hospital.

mRNA and protein isolated and amplified from single cells

mRNA isolation and amplification from single cells. The quantity of mRNA in a single cell is

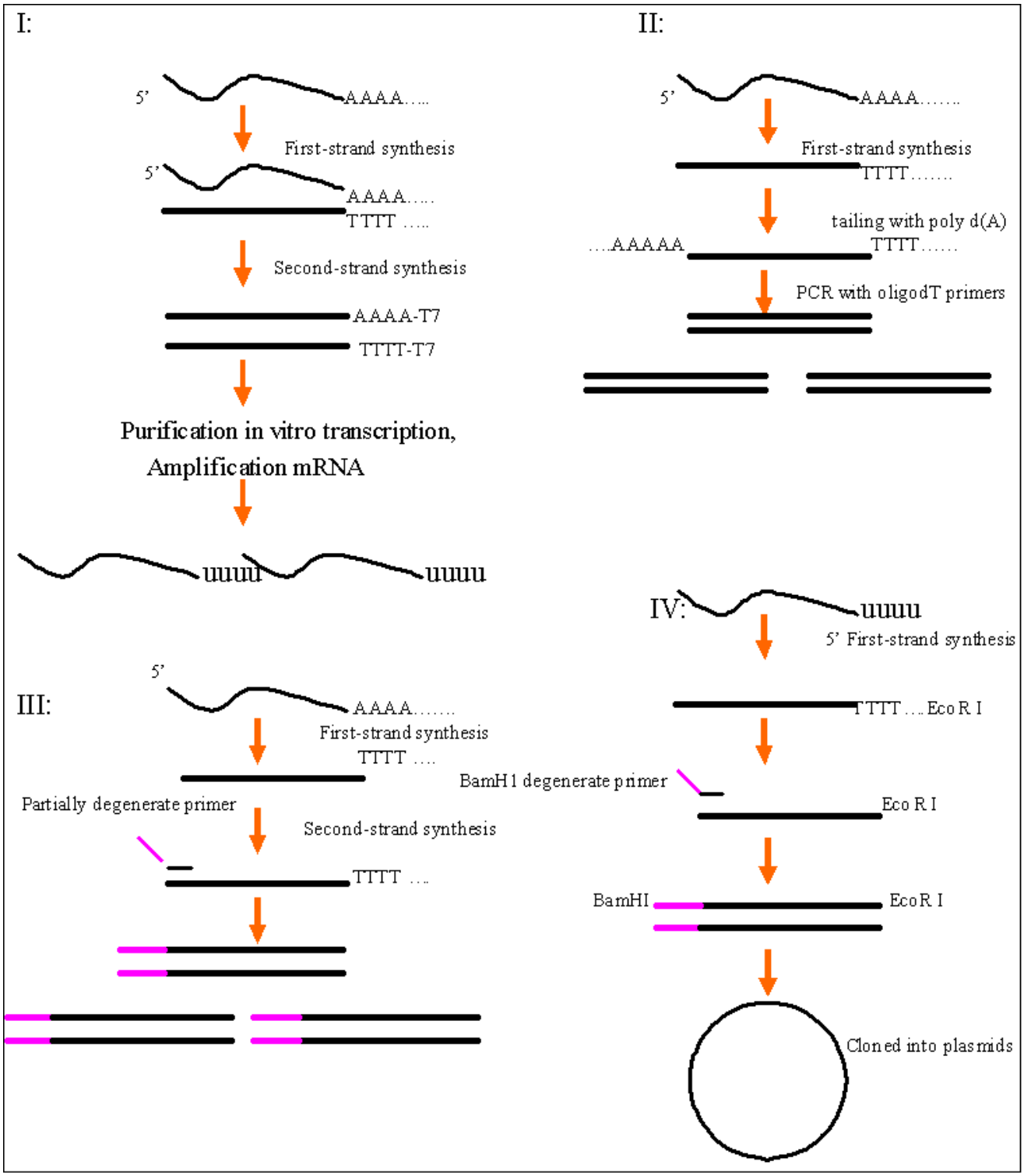

Figure 2. mRNA amplification from single cells.

approximately 1.0 pg (about 5 x 10^5 molecules) (Ambion, 2004). Although some scientists try to isolate RNA from single cells, we prefer to use a crude cell lysate without purifying procedures (Klebe et al. 1996). This protocol has two important advantages. First, it ruptures the cells and releases the RNA directly into a cell lysis buffer without loss of RNA. Moreover, the heating step to rupture cells inactivates endogenous RNase, further protecting RNA from degradation. Theoretically, in order to observe subtle differences in parallel gene expression, genome information

amplification should be applied in single-cell studies. At present, there are two strategies to employ genome information amplification: mRNA amplification (aRNA) and PCR-based cDNA amplification.

The aRNA procedure begins with total RNA or poly(A) RNA that is reverse transcribed using an oligo (dT) primer containing a T7 RNA polymerase promoter sequence. After first-strand synthesis, the reaction is treated with RNase H to fragment the mRNA. These fragments serve as primers during a second-strand synthesis reaction that produces a double-stranded DNA template for transcription. rRNA, mRNA fragments and primers are removed before using the cDNA template to produce linearly amplified aRNA. The amplification yields can reach 1000-5000 fold following two rounds of *in vitro* transcription (Figure 2-I). RNA amplification and is commercially available and has been increasingly reported in gene expression studies (Eberwine, 1996).

PCR-based amplification has two protocols: specific profile and global profile applications. Specific profile methods such as rtPCR or multiplex rtPCR reactions are sensitive at the single-cell level, especially in nested PCR. Because the genes studied this way are pre-selected, it can only be applied to known genes. In order to overcome the problem, global PCR-based approaches have been developed in genomic analysis. One approach is homomeric tailings, and another is 3′-(3-primer-end) amplification (TPEA). The former (Figure 2-II) (Toellner et al. 1996) uses terminal deoxynucleotide transferase-generated homomeric 3’ tails to the first-strand cDNA. After rtPCR and 3’ tailing addition and PCR amplification, it has been applied to the analysis of single-cell global gene expression. Even though homomeric tailings can be used effectively in global profile analysis, many of the cDNA copies are not full length and shorter cDNAs are preferentially amplified. 3-primer-end-amplification (TPEA, Figure 2-III) (Dixon et al. 1998) is a partially randomized amplification of mRNA using an oligo-dT primer together with a 5’ primer containing a random pentamer. It can enable the detection of both high- and low-abundance mRNA transcripts from single cells. Because TPEA also has a 3’ bias, full-length cloning is more difficult. Moreover, only 40 to 50 genes can be analyzed in samples derived from single cells.

We have also developed a more facile strategy to screen the genome at the single cell level. To illustrate, three techniques (RNA directly from cell lysis, randomized primer design as differential display and single cell genome cloned into plasmids) are simultaneously combined (Li et al. 2000). Following cell lysis and reverse transcription PCR, a 3’ end oligo $(dT)_n$ primer and a set of 5’ end arbitrary primers (both containing restriction enzyme terminals) are used in an amplification by PCR. After double digestion, the genome from a small number of cells is introduced into plasmids and transformed into cells. As Figure 2 and Figure 3 indicate, subtractive hybridization from a reference cell genome is employed in the modified method so that artefacts of cDNA amplification from test cells are minimized (Li et al. 2002). The technique has been used in genome expression analysis from 10 to 100 cells. The advantage is that the expression results are very sensitive and accurate because they exclude problems of artefacts. The disadvantage is similar to TPEA, that is, expression has a 3’ bias (Figure 2-IV). Over the past two years, as aRNA techniques have been developed, cDNA detection sensitivity has significantly increased so that we can use either cells from microdissection or single cells obtained from culture to analyze their genome expression (Figure 3) (Zhang et al. 2004).

Protein signal amplification from a single-cell. Traditional methodologies for protein detection and quantification include two-dimensional gel electrophoresis, mass spectrometry, and antibody (Ab) binding. As we discussed previously, the application of these traditional proteomics-oriented technologies at the single-cell level has been limited because each methodology needs relatively large amounts of tissue.

Table 2. Single-cell sampling.

Methods	Advantages	Disadvantages
Flow-cytometric cell sorting	Large quantity of cells; rapid separation	Limit in some cells such as neuron without microenvironment data
Laser-assisted mechanical microdissection	Very little contamination with microenvironment data	Laborious
Laser-catapult microdissection	Very little contamination with microenvironment data	Special slides
Laser-capture microdissection	Very little contamination with microenvironment data	Theoretical damage to the target cell

The development of protein arrays using antibody-binding technology has presented a new opportunity to study protein expression at the single cell level. Recently, two protein array techniques have rapidly been extended. The first is the use of intact antibodies, antibody fragments (single-chain fragment variable (ScFv) fragments) or exocyclic peptide-based complementarity determining region (CDR) subunits as antigen detectors. The second is the Ab used as amplification signals. In order to detect protein and peptide molecules, several Ab signal amplifications have been successfully employed in order to improve sensitivity. For example, immuno-PCR and T7 RNA amplification have been reported in several journals. In the former, the PCR technology is combined with

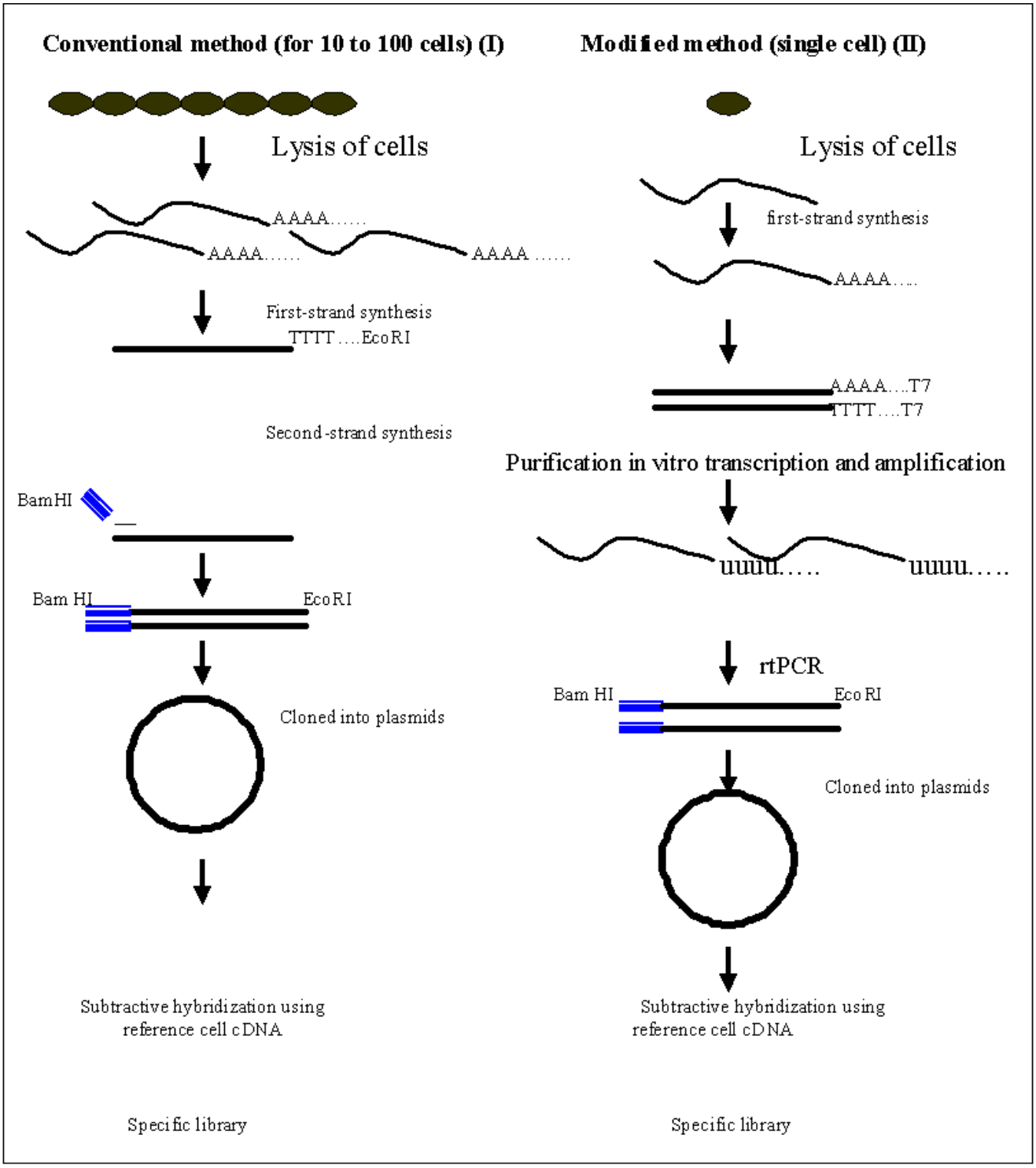

Figure 3. A strategy of genome library from single cells.

conventional immuno-detection methods as shown in Figure 4(A) (McKie et al. 2002). Streptavidin is added to a biotinylated Ab-antigen complex whereupon a known biotinylated-DNA fragment is added, resulting in the formation of a specific antigen-Ab-DNA conjugate. The attached marker DNA can be amplified by PCR with appropriate primers. Some results have shown that ~10^5-fold increase in sensitivity over an alkaline phosphatase-conjugated ELISA is obtained. This approach, with slight modifications, has been used to detect a variety of antigens, including a human protooncogene protein and tumour necrosis factor α.

Although the immuno-PCR technique has some advantages over traditional methods of protein detection, such as increase in sensitivity, there still exist several notable limitations to its use. One of the major limitations of immuno-PCR lies in the nonlinear amplification ability of PCR, which limits this technique as a quantitative detection method. Some of these problems have been overcome with a relatively isothermal rolling circle DNA amplification technique (RCA). As demonstrated in Figure 4(B) (Zhang et al. 2001), T7 RNA amplification resolves these problems and shows a linear relationship between protein expression and an expression indicator such as luciferase.

Methods and application of genomic expression from single cells

Methods of genomic expression from single cells.

Single-cell gene expression analysis can be carried out both at the specific profile and global genome profile. *In situ* hybridization and rtPCR belong to the specific profile. *In situ* PCR combined with immunohistochemical detection is frequently used as a measurement of single-cell gene activity (Gey et al. 1999). Multiplex rtPCR is also effective for observing gene expression at the single-cell level (Hahn et al. 2002). At present, rtPCR using real-time detection of PCR products can quantify gene expression at the single-cell level with reduced risk for artefacts resulting from contamination or illegitimate transcript amplification (Liss, 2002). However, because the primers are pre-selected, expression profiles will not contain previously unreported transcripts or novel sequences.

Global genome profile expression analysis at the single-cell level holds new promise to analyze disease pathogenesis and tumorigenesis. At present, four techniques are utilized to advance the global genome profile of a single cell (in addition to the previously described specific profiles such as *in situ* hybridization and multiplex rtPCR). These global genome profiles include differential display, subtractive cloning, microarray, and protein array. As shown in Figure 2 and Figure 3, differential display and subtractive cloning can be employed with a small number of cells in which the resolution is from one cell to 10^4 cells (Chen and Talmage, 1999). Because both of these methods may have an artefact contamination after amplification, it may result in variable genome expression at the single-cell level. As discussed above, we introduced a strategy combining amplifying RNA, randomized primers (with restriction terminals for cloning into plasmid) and subtractive hybridization (for eliminating some artefacts), which has successfully been used in genome expression at single-cell level. Although our method still has a problem in screening the genome, that is, some 3' bias, after sequencing the genome at 3' terminal fragment, GenBank analysis can allow us to eventually determine full-length genome prediction (Li et al. 2000; Li et al. 2002; Zhang et al. 2004). Here, some explanation and details shown as above (such as including A: designs of random primers with restriction enzyme site, B: cDNA amplification, C: cloning into plasmid and storage of library and D: subtractive hybridization using reference cell cDNA), we illustrate the basic protocol in

Table 3. Functional genomics analysis for single cell or small cell number.

Methods	Minimum Cell	Throughput	Sequencing	Application	Disadvantage
rtPCR base					
Multiplex rtPCR	single cell	poor	N/A	specific gene	one cell <24 genes
In situ rtPCR	single cell	poor	N/A	specific gene	one cell <5 genes
Gel base					
DD	single cells	medium	low	Parallel gene expression	false positive
Colony base					
Subtractive cloning	10^0-10^4 cells	medium	low	Parallel gene expression	not typical parallel
Hybridization base					
Microarray	single-cell developing	High	N/A	Parallel gene expression	no sensitivity
Protein base					
Protein array	single-cell developing	High	N/A	Parallel protein expression	no sensitivity

Figure 5.

DNA microarrays used for single-cell DNA have emerged. One method involves modifying some procedures to increase resolution such as aRNA and cDNA amplification, and another involves remodelling microarray platform materials. Because modified microarrays still suffer from inadequate resolution, other single-cell rtPCR or DD are required to confirm the results (Bahn et al. 2001). In remodelling microarray platform materials, recently, a high-density fibre optic DNA microarray has been developed, in which there are 6,000 to 50,000 fused optical fibres, and each fibre terminates with an etched well. This array platform provides many advantages over other array formats. These microarrays contain the smallest feature sizes so that it is believed that the fabrication protocol enables, in the future, their expansion into single cell-based assays (Epstein et al. 2003).

Protein arrays based on antibody-binding technology shall emerge by development of immuno-PCR and T7 RNA amplification to screen protein expression at the single cell level. If intact Abs, ScFv fragments or exocyclic peptide-based complementarity determining region (CDR) subunits can serve as antigen detectors in the protein array, it can likely facilitate the development of a robotic platform for proteomics. If a large scale of antibodies can be produced, the usage of this approach will have a good potential.

Application of genomic expression from single cells. Gene expression profiles of single cells are providing tremendous insights into disease pathogenesis, especially in tumorigenesis. Eberwine reported gene expression in a single live neuron, and a successful application involving rtPCR appeared (Eberwine, 1992; Eberwine, 1996). The specific profile has extended from single-gene expression to multiplex PCR, from regular expression to *in situ* rtPCR, and from routine rtPCR to real-time rtPCR. For instance, some scientists recently reported that computational fluorescence microscopy with multiplex probes can analyze the expression patterns of eleven genes in individual serum-stimulated cultured cells (Levsky et al. 2002).

For global profiles, the progress is very slow due to current technological limitations. Most scientists screen global profiles, as discussed above, by using microarrays from a large number of cells and then confirming them by analyzing the specific profile at the single-cell level by using rtPCR or differential display. For example, differential display was first utilized in the profile of a single CD3 positive cell in 1998 (Renner et al. 1998). A single sea urchin egg was initially analyzed *via* microarray in 1998 (Michael and Walt, 1999), and later, DNA microarray was used to analyze the profile of a single plant cell (Brandt et al. 2002). In 2000, we reported a strategy to observe the global profile of a small number of T-cells (10 to 100 cells) (Li et al. 2000). Recently, some investigators have suggested using tiny tissue samples from laser-captured microdissection to analyze genomic profiles, especially in complex multifactorial diseases such as neuropsychiatric disorders (Ginsberg and Che, 2002). According to the idea of micro-dissection to obtain the tiny tissue or single cell, a genomic change of aberrant crypt foci have been successfully observed in our laboratory (Li, 2002).

FUTURE OF GENOMICS AT THE SINGLE-CELL LEVEL

Although technical developments and clinical application in single-cell gene expression have been established and developed, the techniques and applications still need to be optimized. A mature genome expression analysis at the single-cell level needs the following: (1) a good method for

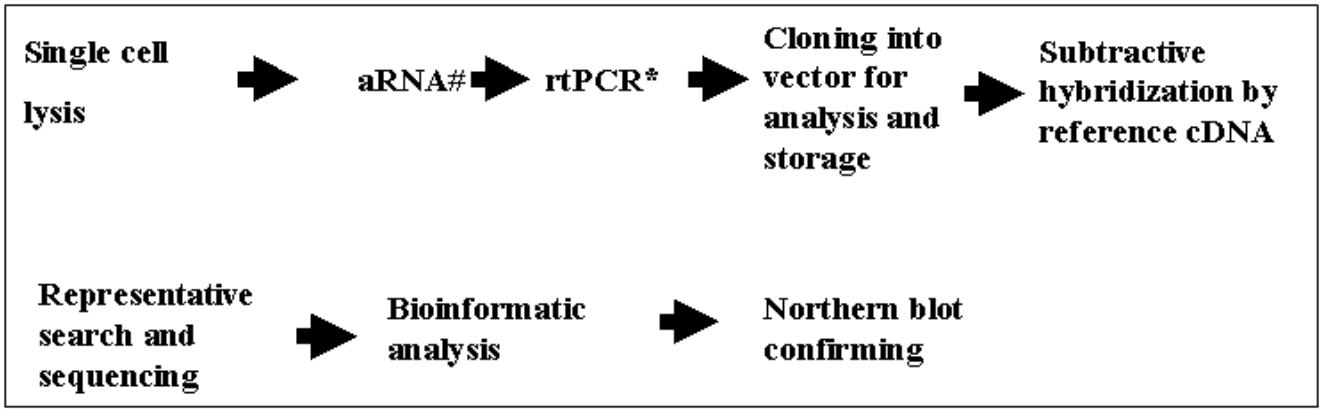

Figure 5. Genomic expression analysis at single cells.

shown at Figure 3.
* illuminated at Figure 2IV.

isolating pure homogenous cells; (2) intact bio-molecule harvest for mRNA and proteins along with a high-fidelity amplification system; and (3) a sensitive method to detect the biomarker at the single-cell level. As we indicated above, no single method can currently satisfy all of these requirements. For example, *in situ* hybridization and multiplex rtPCR are limited to the analysis of a known profile. Also, DNA microarrays require the development of more sensitive platform materials. In other words, after developing immuno-PCR and T7 RNA amplification, it is possible to screen protein expression at single cell levels, but production of many thousands of intact protein Abs and peptide Abs still face great challenges. We have designed randomized primers and combined strategies including amplifying RNA, cloning into plasmids and subtractive hybridization to minimize some artifacts, and this strategy has been successfully applied from several hundred cells to a single-cell level. However, the problem with 3' bias still needs to be resolved.

Fortunately, many scientists and companies are focused on overcoming these challenges. It is believed that single-cell global genome profiling will become an important tool for scientists and physicians to study pathogenesis, early clinical diagnosis and treatment in the near future.

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Note: This paper for a memory of Dr. Harvey D. Preisler, who contributed his whole life to study tumorigenesis of AML, He was a director of Rush Cancer Institute from 1990 to 2002.