A Method for the Highly Parallel Analysis of Gene Expression of Single Cells

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ABSTRACT

Presented is a method for carrying out rapid parallel single cell gene expression analyses on large numbers of cells in parallel. The analysis is carried out on a DNA microarray using a microfluidic device. Microfluidics is used to separate and trap the cells, but also to ensure quantitative hybridization of the individual cells mRNA content on the microarray. Fluorescent labels are incorporated following hybridization using primer extension. An in-house built microarray scanner with single molecule resolution is used to image and count individual molecules. Thus the gene expression profile of individual cells can be determined quantitatively and in a parallel fashion. In this paper we present results on cell capture, mRNA capture, labeling and single molecule counting.

Keywords: microfluidics, microarrays, hybridization, DNA, cells

1 INTRODUCTION

There are many methods for biochemical characterization of cells and tissues. Analyses are most often carried out after lysing cells to release their contents. Because it is difficult to isolate single cells and because normal methods of detection are not sensitive enough to measure the contents of single cells, it is usually necessary to use a large number of cells. This "blender approach" results in an average picture of the cells that are analyzed [1]. Furthermore, the greater the number of cells within the sample, the greater the risk that crucial information present in just a few of the cells is overlooked. For a complete description of the state of any biological system, therefore, it would be advantageous to analyze individual cells.

Fluorescence in situ hybridization (FISH) permits the study of the expression a few genes in thousands of individual cells. Using this method, for instance, it has been demonstrated that considerable cell-to-cell variability exists at the gene expression level, even among those cells obtained under synchronized culture conditions [2]. However, because the total number of mRNA molecules expressed in a cell typically lies between 6,000 and 10,000, this technique does not adequately give information regarding the genetic diversity of the cells [3].

One solution is to use advanced PCR methods such as global amplification PCR and three-prime-end amplification PCR. The latter, in particular, allows the expression of up to 40 genes at the single cell level to be determined [2]. A disadvantage of these methods is that the data obtained is not quantitative and often skewed. Even though gene expression at the single cell level can be measured in principle using these methods, the task of separating the cells and using PCR on the contents of each individual cell is laborious and time consuming.

Recently, Marcus et al. [4] have addressed this issue by constructing a microfluidic device that allows PCR to be performed on a number of samples in parallel that may contain as few as one cell per sample. They demonstrate how four such samples can be analyzed in parallel, and do not rule out the possibility of scaling this up to as many as 500 samples. One difficulty, however, appears to be in obtaining just one cell per sample.

2 EXPERIMENTAL

The principle of operation is shown in Figure 1. The device consists of a set of microfluidic channels running perpendicular to a linear DNA microarray (i.e. a microarray where the features are lines, rather than spots), targeting specific genes. The main steps in the process are cell capture, lysis, mRNA capture, labeling and imaging. As a result of this configuration, the content of each cell is interrogated using the same sequence of microarray features.

2.1 Cell Capture

The microfluidic device we use has four ports (see Figure 2A). Of these four ports, one is a bifurcating inlet port, and one a merging outlet port. The two other ports connect to a bus line (see Figure 2B) which runs between the cell trapping sites and the main inlet. The bifurcating inlet and bus line are used to introduce cells and reagents. The device contains 256 trapping sites, each one of which is connected to a capillary channel. The 256 capillary channels run parallel to one another, and converge into the main outlet port. The cell trapping sites are funnel shaped, with a funnel mouth comparable to the dimension of the particular cell type (e.g. mouse myeloma cells). Flow control at this stage of development is purely hydrodynamic.

2.2 mRNA Capture

Following separation and trapping (see Figure 2C), the cells are lysed in parallel, and the content of each cell is transported through a capillary channel across the linear array (see Figure 1C-D). The transport of the cell content is carried out at a slow, controlled flow rate in order to ensure complete hybridization. It has been shown that hybridization times can be reduced using microfluidic devices, due to shorter diffusion distances in microfluidic channels [5, 6]. We use the same underlying principle to achieve quantitative hybridization, i.e. quasi-complete (>99%) capture of mRNA molecules, which is achieved by use of a low flow rate (see Figure 4). Complete or quasi-complete capture of mRNA is essential given the small copy numbers of certain genes in single cells (e.g. 10 - 100 copies).

2.3 Labeling

Following capture of the mRNA the array is separated from the cell capture device and washed to remove cellular contents (Figure 1F). The slide with the array and captured mRNA molecules is immersed in a standard reverse transcription mix. The reaction mixture includes Cy3 labeled dNTP for incorporation and detection.

As well as allowing the mRNA to be captured directly to the array without the need for any sample preparation, on-array reverse transcription also offers the advantage of higher specificity over hybridization alone: mismatches at the ends of the hetero-duplex prevent primer extension via reverse transcriptase [7], and thus incorporation of labels, while they are significantly less destabilizing in conventional hybridization experiments. After the reverse transcription, the arrays are washed and scanned.

2.4 Single Molecule Detection and Counting

The ability to detect single molecules has been demonstrated on microarrays, as discussed in a recent publication [8]. A key prerequisite of the technology is the use of high spatial resolution. However, the instrument discussed in [8] requires that the common microarray substrates (1mm thick) are replaced by fragile cover slips (a mere 170µm thick). We have developed an instrument with an alternative design that is capable of scanning conventional microarrays.

Scanning a microarray at high resolution and sensitivity brings many benefits for conventional gene expression analysis on microarrays. In [8], Hesse's group showed that the sample amount could be reduced by a factor of about 100 without amplification, avoiding bias introduced by the amplification process and reducing the time and complexity of sample preparation. Importantly, the dynamic range of the analysis is extended at both the low and the high end of the range; there is potential for a gain of two to three orders of magnitude. The same advantages can be expected from any instrument achieving single molecule detection at high spatial resolution.

As has been discussed in [9], counting of single molecules hybridized to a microarray can be automated. This provides the ultimate sensitivity a microarray reader can exhibit: of the two sources of noise contributing to the microarray experiment, namely read-out noise of the scanner (electronic noise, thermal noise of the detector, quantization noise, etc.) and biological noise (non-specific binding, incomplete washes, etc.), the instrumental component can be eliminated.

3 CONCLUSIONS

In this paper, we have presented progress towards the development of a device for carrying out rapid single cell gene expression analyses on a large number of cells in parallel. In particular, we have demonstrated cell separation and capture, quantitative hybridization of mRNA to a microarray, in-situ labeling using reverse transcription and imaging at single molecule resolution.

ACKNOWLEDGEMENTS

Part of this work (single molecule detection and counting) was supported by the EU-FP6 integrated project MolTools.

REFERENCES

[1] M.E. Lidstrom and D.R. Meldrum, Nat. Rev. Microbiol., Vol. 1, September 2003, pp. 158 - 164

[2] J. M. Levsky and R. H. Singer, Trends in Cell Biology, 2003, 13(1), 4-6

[3] A.K. Dixon, P.J. Richardson, R.D. Pinnock and K. Lee, Trends Pharmacol Sci. 2000 Feb;21(2):65-70.

[4] J.S. Marcus, W.F. Anderson and S.R. Quake, Anal. Chem. 2006, 78, 3084-3089

[5] J. H.-S. Kima, A. Marafie, X.-Y. Jia, J.V. Zoval, M.J. Madou, Sensors and Actuators B 2006, 113, 281-289

[6] J.A. Benn, J. Hu, B.J. Hogan, R.C. Fry, L.D. Samson, T. Thorsen, Analytical Biochemistry 348 (2006) 284–293

[7] N. Milner, M.Sc. thesis, Oxford, 2005

[8] Hesse et al., Genome Research 16:1041–1045 (2006)

[9] L. Muresan, B. Heise, E.P. Clement and J. Kybic, IEEE International Conference on Image Processing, 11-14 Sept 2005. Volume 2:1274 – 1277.

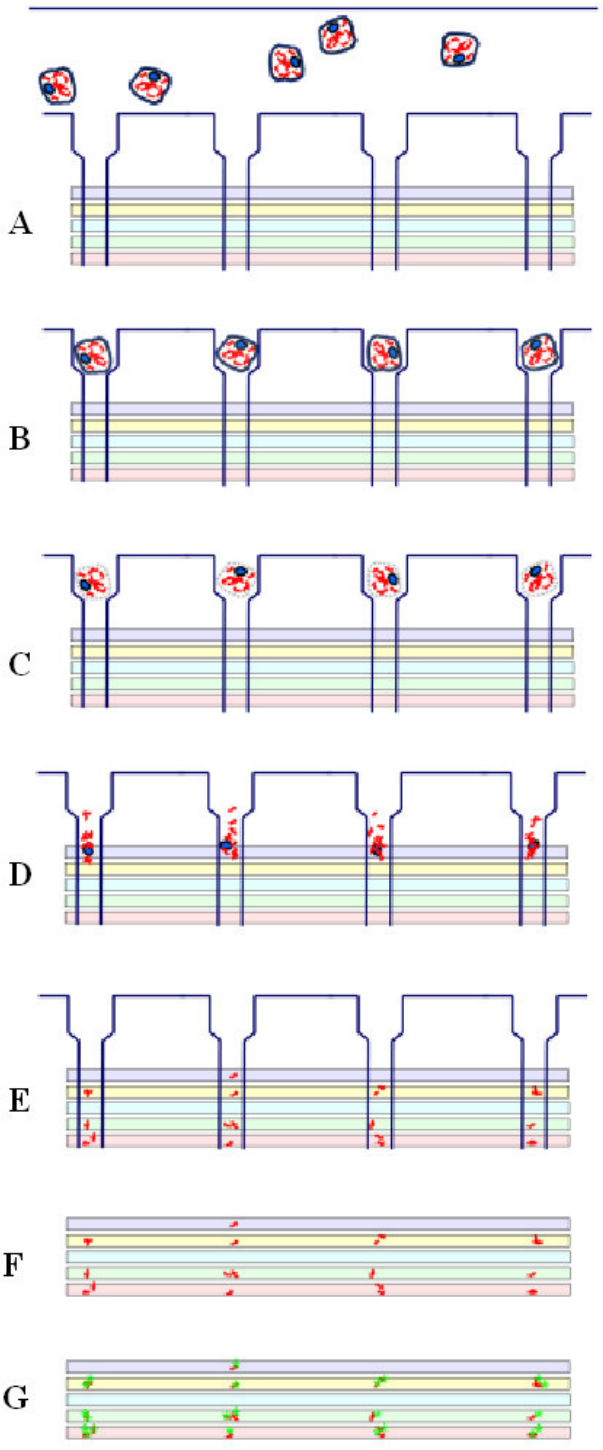


Figure 1: Schematic representation of a cell analysis device: a microfluidic device consisting of capillaries connected to cell trapping sites is applied to a microarray consisting of lines of probes (different represented by different colors). **A**-**B**: Cell capture using trapping sites (funnel shaped); **C**: Cell lysis in funnels; **D**: mRNA transport in capillaries; **E**: mRNA capture on probes; **F**: disassembly; **G**: labeling using reverse transcription

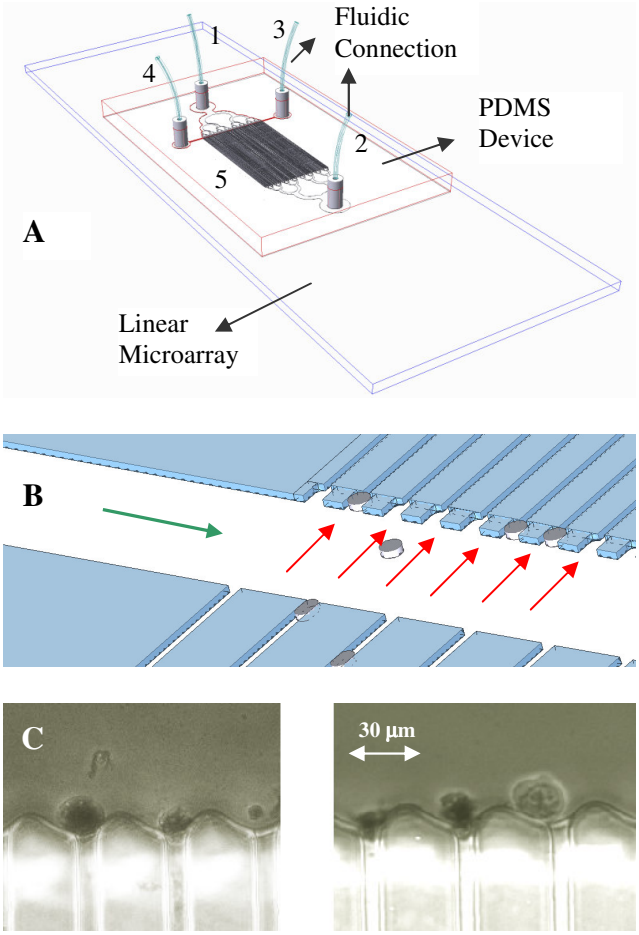


Figure 2: Schematic representation of the device. **A**: PDMS structure with fluidic connections is applied to a linear microarray (1: main inlet, 2: main outlet, 3: bus inlet, 4: bus outlet, 5: capillary channels); **B**: Close up of the bus line, showing part of the bifurcating main inlet, the cell docking sites connected to capillary channels, the direction of capture (red arrows) and the direction of reagents (green arrow); **C**: images of docked cells inside the device

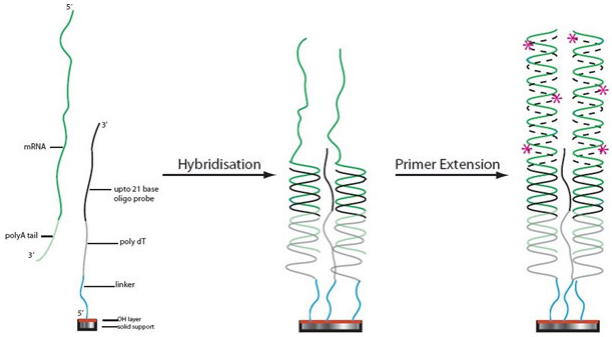
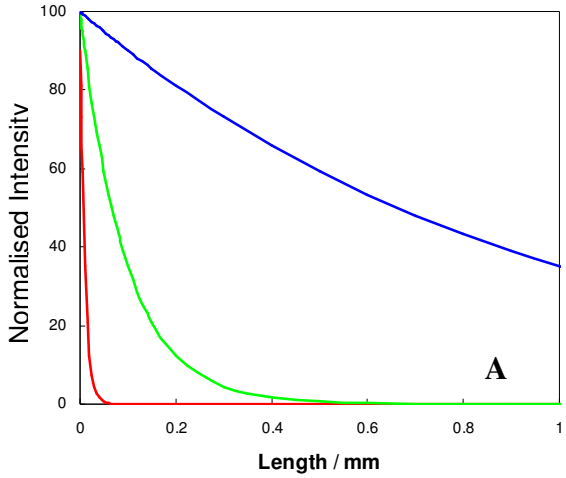


Figure 3: Principle of labeling using primer extension: an mRNA molecule (target) hybridizes with an oligo-nucleotide (probe) attached to a surface (microarray); following hybridization, the probe is extended using a reverse transcriptase enzyme, incorporating fluorescently labeled nucleotides.

NSTI-Nanotech 2007, www.nsti.org, ISBN 1420061844 Vol. 3, 2007



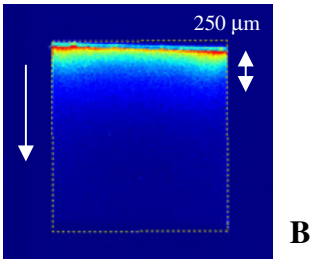
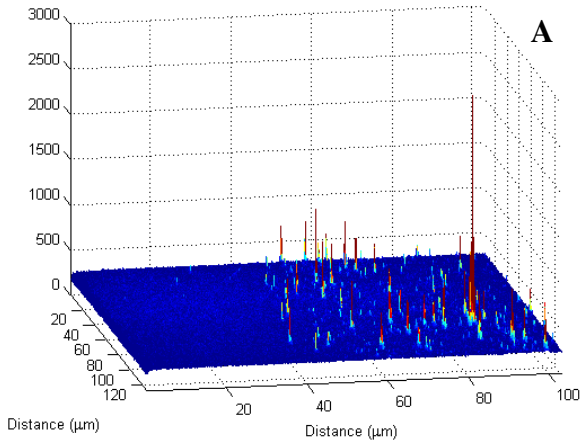
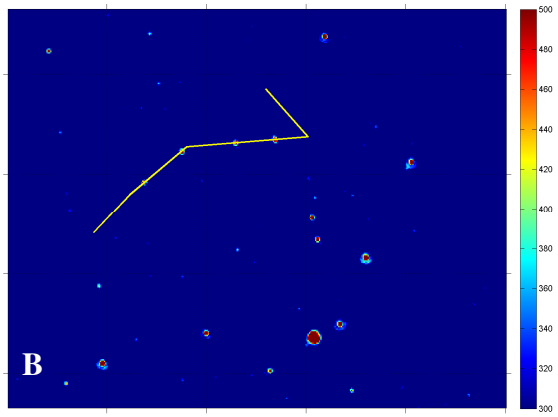


Figure 4: Demonstration of quantitative pick-up of RNA molecules. **A**: Theoretical calculation of hybridization (represented by drop in concentration in solution) in a 25 µm deep channel as a function of distance traveled by an RNA molecule (1200 nt) across a microarray feature at different controlled flow rates (blue: 20 µm/s; green: 2 µm/s; red: 0.2 µm/s). It can be seen that at 0.2 µm/s, hybridization is quasi complete within a distance traveled of less than 100 µm. **B**: Experimental data: false color images showing quasi-complete hybridization at 0.5 µm/s in a 25 µm deep channel within the first 300 µm of a 1 mm2 microarray feature.





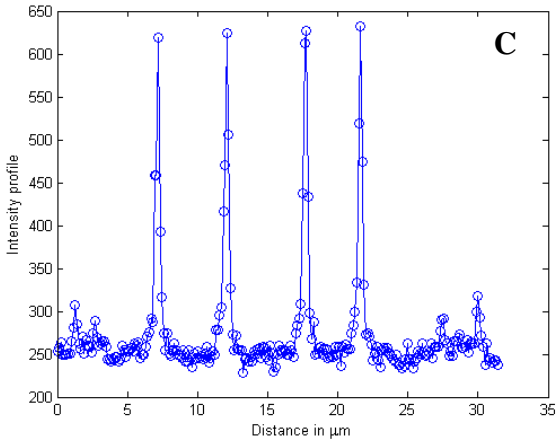


Figure 5 **A**: Pseudo-3D plot of the edge of a patch. To the left, the absence of hybridization is evident, whereas on the right there are mainly identical peaks, identifying single molecules. There is, in addition, a higher peak, which is due to the hybridization of several molecules within the diffraction limited spot. **B-C**: line profile through some randomly selected peaks. It is clear that the signal height is similar or identical, and that it can reliably be distinguished from background.