19th International Symposium on Laboratory Automation and Robotics Proceedings, October 2001 Boston MA.

DNA microarray fabrication and processing: Automation in the laboratory.

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ABSTRACT

The confluence of robotics, biotechnology, computer sciences and the completion of genome sequencing efforts for several organisms, have resulted in revolutionary changes in how biomedical research is carried out. It is now possible to fabricate high-density arrays of specified DNA sequences that include every known gene of an organism on a single glass slide. Labeled RNA or DNA targets (such as messenger RNAs obtained from cells, tissues or organisms under different conditions) can be analyzed by hybridization on the array. Differences in the levels of expression for thousands of genes can thus be assessed all at the same time in a single, simple experiment. Genomics, informatics and automation are playing increasingly important roles as discovery tools in the basic biological sciences, and as diagnostic and rational therapeutic aids in the clinical arena. We discuss the use of automation to increase productivity in microarray fabrication and describe how automated procedures increase the quality of results in microarray experimentation.

Introduction

Microarray technology has made it possible to evaluate in parallel the expression of thousands of genes. Microarray technology utilizes a robotic spotting device or "microarrayer" to print DNA sequences onto a solid support typically derivative glass slides or membranes (I). The DNA probes immobilized on the microarray slide as spots, can either be cloned cDNA or gene fragments (ESTs, expressed sequence-tags), or oligonucleotides corresponding to known genes or putative open reading frames (ORFs). The arrays are hybridized with fluorescent targets prepared from cellular RNA and the fluorescent signals Cy3 and Cy5 labels are determined. The standard microarray experimental platform consists of comparing mRNA abundance in two different samples. One fluorescent target is prepared from control mRNA and the second from mRNA isolated from the treated cells or tissue under investigation. Both targets are mixed and hybridized together on the same microarray slide and target gene sequences hybridize to their complementary sequences in the spots. The microarray is excited using a laser and the fluorescent intensity of each spot is determined. The relative intensities of the two colored signals on individual spots are proportional to the amounts of specific mRNA transcripts in each sample, enabling an estimation of the relative expression levels of the genes in the sample and control populations (II).

Spotted arrays are highly versatile and can make use of a wide variety of clone sets or oligonucleotides. Microarrays thus enable individual investigators to perform large-scale analysis of model organisms and to customize arrays for special genome applications. The choice of probe oligonucleotide or cDNA is determined largely by several factors namely cost, commercial availability and the experimental question being addressed. Many genome array sets are available as commercial oligonucleotide sets or printed slides whereas others must be fabricated. In this manuscript we address the use of automation in increasing the productivity of microarray fabrication and describe how automated procedures increase the quality of the data.

EXPERIMENTAL

Mouse Microarray Preparation Workflow

The mouse GEM 1 Clone List (build 35) containing 8,734 clones was purchased from Incyte Pharmaceuticals (Palo Alto, CA). A Qpix colony-picking robot was used for picking additional bacterial colonies into 96 well plates. Bacterial clones containing cDNA inserts for growth and replication were inoculated using a pin replicator (VP Scientific) into 96 well Falcon U-bottom plates (Nunc) containing LB/Ampicillin (50 µg/ml) and incubated overnight at 37ºC with agitation. PCR reactions (150 µl) were assembled in 96 well microtiter plate format using a Qiagen Biorobot 3000. PCR was performed with MJ Research Tetrad thermocyclers. Each reaction mix consisted of 10 µM dNTP, 20 µM primer mix (forward primer 5'-ctg caa ggc gat taa gttgggtaac-3' and reverse primer 5'-gtg agc gga taa c aatttcacacaggaaacagc-3'), 3µl of overnight culture and 3.75 U MasterTaq (Brinkman Eppendorf). Cycling conditions were as follows; an initial five minute denaturation at 95^0C to lyse the cells and release the plasmid DNA, 35 cycles (94^0C for 1 minute, 58^0C for 1 minute, 72^0C for 4 minutes) followed by 10 minutes at 72^0C. One fifth of the PCR amplification was analyzed on a 0.8% agarose gel containing ethidium bromide and visualized with an AlphaImager 2200 Documentation and Analysis

system (Alpha Innotech). Electrophoretic gel analysis was carried out using gel trays purchased from BioRad. Gel loading was carried out manually with a twelve-channel pipettor. PCR products were purified on a Biorobot 3000 (QIAGEN) using the QIAQUICK 96 PCR BioRobot Kit. The yield of PCR product was assayed using the Picogreen dsDNA quantitation kit (Molecular Probes) and a Bio-Tek FLX800 microplate fluorescence reader.

Preparation of fluorescently labeled cDNA.

1 µg of RNA (mouse spleen poly A^+ RNA and a synthetic RNA target amplicon created from plasmid vector sequence respectively) were converted into fluorescently labeled cDNA by incubation with an oligo dT primer (44 µM final) at 70 °C for 10 minutes in a final volume of 9 µl. The solution was then mixed with 11 µl of a solution 1.8X first strand buffer, containing 18 mM DTT, 900 µM dATP, 900 µM dGTP, 900 µM dTTP, 230 µM dCTP, 230 µM Cy3- or Cy5-dCTP (APBiotech, UK), 20 U of Rnasin (Promega), and 200 U Superscript II reverse transcriptase (LifeTechnologies: Cat# 18064-014). The reaction was incubated at 42 °C for 2 hours and heated at 100 °C for 5 minutes. RNA was hydrolyzed by the addition of 1 µl of 10 N NaOH and subsequent incubation at 37 °C for 15 minutes. The reaction was neutralized by the addition of 5 µl of 1M Tris pH 7.2 and 2 µl of 5N HCl, and cDNA targets were purified using a Qiaquick PCR purification kit (Qiagen). Typically, one fifth of each reaction was used per hybridization.

Microarray Fabrication, Hybridization, washing, scanning, and data analysis.

PCR products (~10,000) derived from the mouse unigene set and control cDNA clones were printed on reflective slides (APBiotech). DNA clones were heat denatured for 3 minutes at 95 °C, chilled on ice, and arrayed using a Molecular Dynamics Generation III spotter. After printing, the microarrays were allowed to dry completely at ambient conditions. The slides were pretreated with 2xSSPE, 0.2%SDS at 55°C. The cDNA target was lyophilized and redissolved in ~32 µl microarray hybridization solution (25% H_20, 25% hybridization Buffer Version 2 (APBiotech), 50% formamide). For hybridizations performed manually the solution was added to the microarray and a coverslip was applied. Hybridization was allowed to proceed for 14-18 hours at 42°C. The microarray was washed with 1X SSC/0.2% SDS for 5 minutes at 45°C followed by two washes (five minutes each) with 0.1X SSC/0.2% SDS at room temperature. For automated hybridization the cDNA target was resuspended in 150 µl of microarray hybridization buffer (described above) and hybridization and washing were carried out as described above. The microarray was rinsed briefly with water, dried with nitrogen, and scanned using a Molecular Dynamics Generation III scanner. The raw image files were quantitated with the Lucidea Spotfinding software (APBiotech) and data analysis was performed using Spotfire 6.0.

RESULTS AND DISCUSSION

Mouse microarray fabrication.

Figure **1** shows the schematic for the approach employed in fabricating this microarray and the components of this process that are automated. PCR set up, PCR purification, resuspension of the DNA at an appropriate concentration for spotting and reracking of the samples into 384 well plate format were fully automated. Figure **2** reveals the process we have implemented for increased throughput microarray fabrication. The clone set was replicated to maintain viable stocks of this valuable resource. Manipulation of bacteria was carried out in microtiter plate format using a 96 pin-replicating tool (VP Scientific) and with a Qpix colony-picking robot (Genetix). PCR set up was carried out on a Qiagen Biorobot 3000 and the plates were transferred manually to MJ Research thermocyclers for PCR. Gel loading of PCR products was carried out manually with a twelve-channel pipettor. An agarose gel analyses of representative 96 well plate format amplifications is depicted in Figure **2**. In general strong unique amplicons were observed. We classified the results of unigene amplifications as follows; 7997 single amplicons (strong bands), 359 single amplicons (weak bands), 293 multiple bands (typically two or more) and 85 failures. The cDNA clones that generated multiple bands were re-racked using the Qiagen Biorobot and amplified with a nested primer set (data not shown). For the most part multiple bands were still observed, indicating that these cDNAs represent mixed clones. This represents ~3.4% contamination which is below average for amplification of a large clone set.

All of the PCR products were purified on a Biorobot 3000 (QIAGEN) using the QIAQUICK 96 PCR BioRobot Kit. Representative DNA samples from the entire set of amplifications were then quantitated using the picogreen kit (Molecular Probes) to determine an estimate of the yield of purified PCR product. The mean clone total yield was 4955.9 ng. The DNAs were resuspended at a concentration of ~200 µg/ml in 25 µl 50% DMSO-H_20. Many microarray spotters use 200 nl per print run and have a void volume of ~5 µl. Assuming the spotter can print 36 arrays per run, this equates in theory to approximately 3600 microarray slides. The working number of slides however is lower than this due to sample evaporation and other printing variables.

Screening of a mouse microarray with cDNA targets generated from mouse spleen polyA$^+$ RNA and control RNA sequences:

To determine the quality of the microarrays, an experiment was performed to verify that the PCR products amplified using this fabrication scheme generated strong specific signals in a microarray experiment. We printed this set of PCR products representing approximately 10,000 mouse cDNA clones and appropriate controls on a reflective slide (APBiotech). Fluorescent targets were synthesized from mouse spleen polyA$^+$. Additionally a control PCR amplicon target that hybridizes to each array probe was designed. This was used to account for the presence of each array probe, and to more accurately compare hybridization intensities for experiments that incorporated more than one array chip (Rouse *et al.*, in preparation). These targets were used to screen the microarray by conventional manual hybridization (Fig **3**). Green, red and yellow spots are observed which indicates positive hybridization of the labeled targets to the printed PCR products. The enlarged area shows the signal color range observed when using cyanine targets, cye 5 control RNA sequence amplicon and cye 3 spleen. Green (cye 3) and red (cye 5) spots indicate differential hybridization of the targets. The control amplicon hybridization represents quality control of the array as the majority of probes are expected to hybridize to the target. Probes lacking the

amplicon sequence were also spotted as negative controls for amplicon hybridization. Lack of cye 5 signal in probes containing amplicon hybridization sequences indicates a poor quality probe and data from this spot is discounted. Generally strong hybridization signals are observed indicating that the overall quality of the probes is good.

Screening of a *Drosophila* microarray with cDNA targets generated from adult and larval polyA$^+$ RNAs: Comparison of manual and automated hybridization experiments

The final experiment performed was to compare a manual hybridization and an automated hybridization. The microarray utilized for this comparison was a microarray fabricated from Drosophila (fruit fly) cDNA. The Drosophila EST set was acquired as plasmid DNA from the Berkeley Drosophila Genome Project (BDGP). The fabrication details for this array are not described in detail here, but were essentially the same as that for the mouse array. The manual hybridization method was performed in the conventional manner by adding target solution to the array, placing a coverslip on the array and allowing hybridization to proceed manually for 14-18 hours at 42°C. This was followed by washing the microarray with 1X SSC/0.2% SDS for 5 minutes at 45°C followed by two washes (five minutes each) with 0.1X SSC/0.2% SDS at room temperature. The automated hybridization was carried out placing the microarray in the ASP chamber, injecting the target into the chamber using a Hamilton syringe and washing as described above. Fluorescent targets were synthesized from *Drosophila* adult and larval life stage polyA$^+$ RNAs and used to screen the microarray by conventional manual and automated hybridization (Fig **4**). Green, red and yellow spots are observed which indicates positive hybridization of the labeled targets to the printed PCR products. The figure shows the signal color range observed in a portion of the array, when using cye 3 adult and cye 5 larvae targets. Green (cye 3) and red (cye 5) spots indicate differential levels of these particular mRNAs sequences. The cye 5 larval target is much weaker when the manual hybridization is compared to the automated hybridization. This is most likely a result of inadequate mixing of the target due to static hybridization under the coverslip. The mixing effect of the ASP hybridization appears to minimize localized target depletion.

Conclusions

In amplifying large cDNA clone collections for the production of microarrays, great care needs to be taken to minimize sample cross contamination and to ensure the viability of each clone. The use of automation minimizes potential sources of error due to manual intervention. Furthermore when amplifying thousands of clones, the main objective is to minimize the total number of reactions needed to generate sufficient material for printing. A high yield of pure PCR product means plentiful amounts of DNA available for spotting, reduction in cost and labor, and ultimately the production of greater numbers of arrays per PCR reaction. Automation facilitates the achievement of this goal.

The application of automation to the microarray experiment reduces hands-on slide processing time and the number of slide handling steps required. Furthermore the volume of solutions used is greatly reduced. The ASP significantly improves

experimental reproducibility, avoids user inconsistencies, maximizes hybridization efficiencies and enhances signal intensities.

References

(I) Schena, M., Shalon, D., Davis, R.W. and Brown, P.O. (1995). Quantitative monitoring of gene expression patterns with complementary DNA microarray. Science 270:467-470.

(II) Hegde, P., Rong Qi, Abernathy, K., Gay, C., Dharap, S., Gaspard, R., Earle-Hughes, J., Snesrud, E., Lee, N., and Quackenbush, J. (2000). A Concise Guide to cDNA Microarray Analysis. Biotechniques 29: 548-562.

Figure 1

Microarray fabrication. A typical microarray fabrication procedure involves replication of bacterial clones, amplification of cDNA inserts, verification, purification and desssication of the PCR amplicons and finally resuspension of DNA at an appropriate concentration for printing microarrays. The steps that are automated are represented with a Qiagen biorobot icon, and those that are performed manually are depicted with a cartoon of a person.

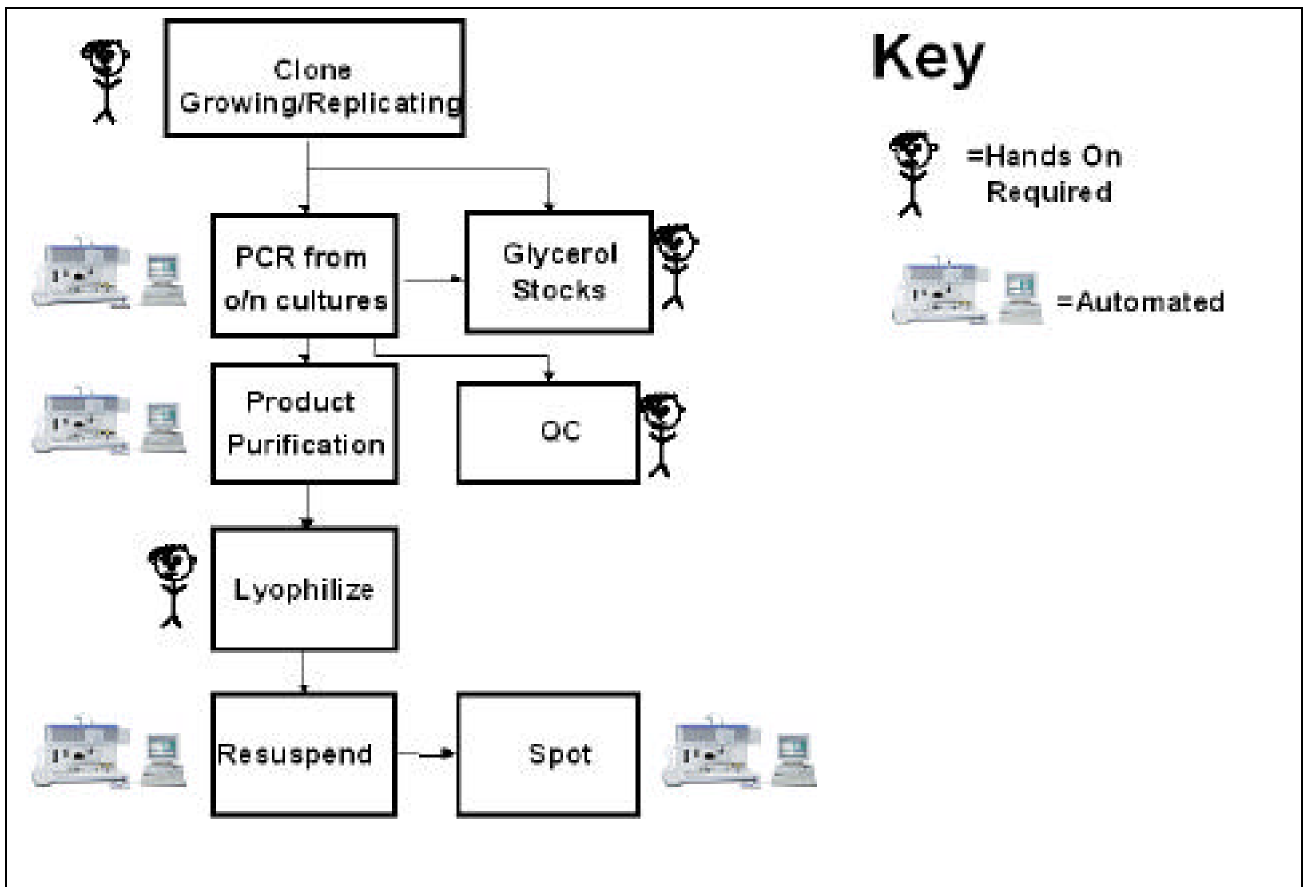

Figure 2
Increasing the throughput of the microarray fabrication process. Automation is employed to aid clone replication, bacterial colony picking and growth, PCR set up and purification and re-gridding the PCR products prior to DNA spotting.

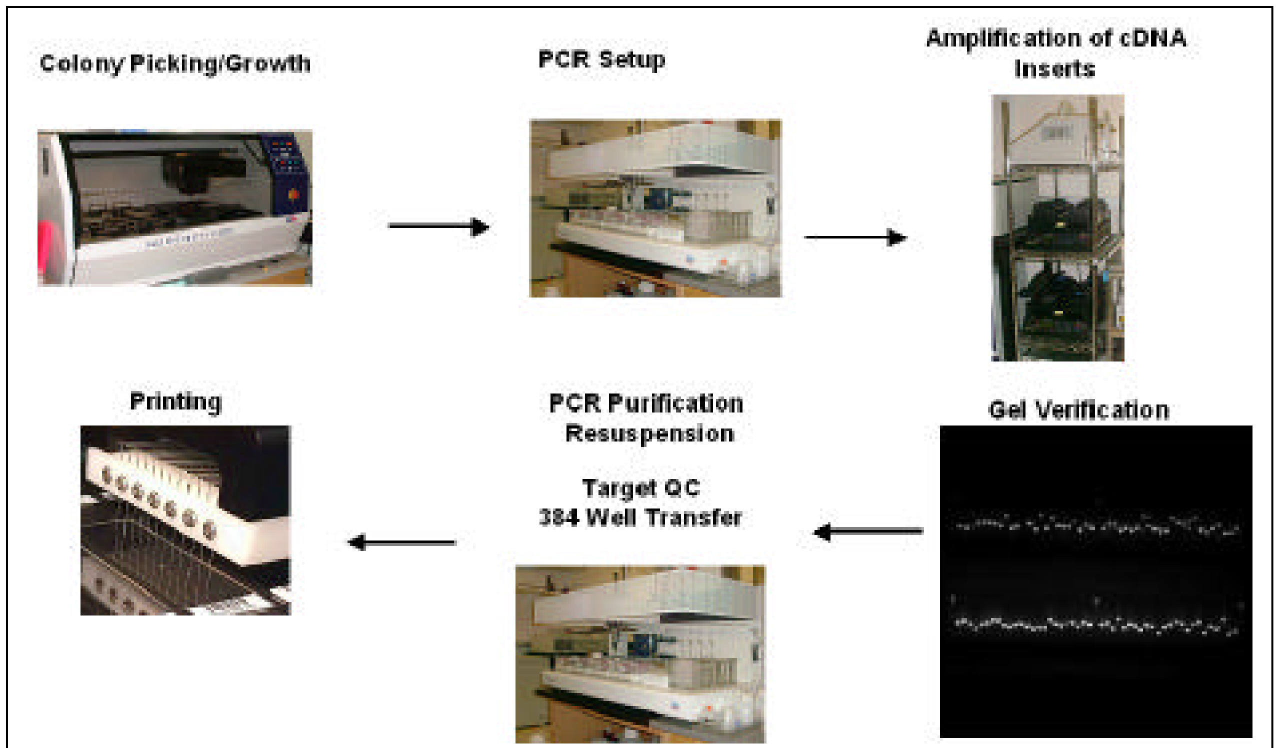

Figure 3
A: Approximately 10,000 cDNA clones derived from the mouse unigene set and controls were printed on Type 7 reflective slides (AP Biotech). Fluorescent targets were synthesized from spleen and control vector sequence (amplicon) RNAs and used to screen the microarray. **B:** The enlarged area shows signal color range when using cyanine targets, cye 5 amplicon and cye 3 spleen. Green, red and yellow spots indicate positive hybridization from the labeled targets. Green (cye3) and red (cye5) spots indicate that the

respective probes are represented differentially in the targets. **C:** Scatter plot of the microarray hybridized signal intensities, cye 5 amplicon versus cye 3 spleen.

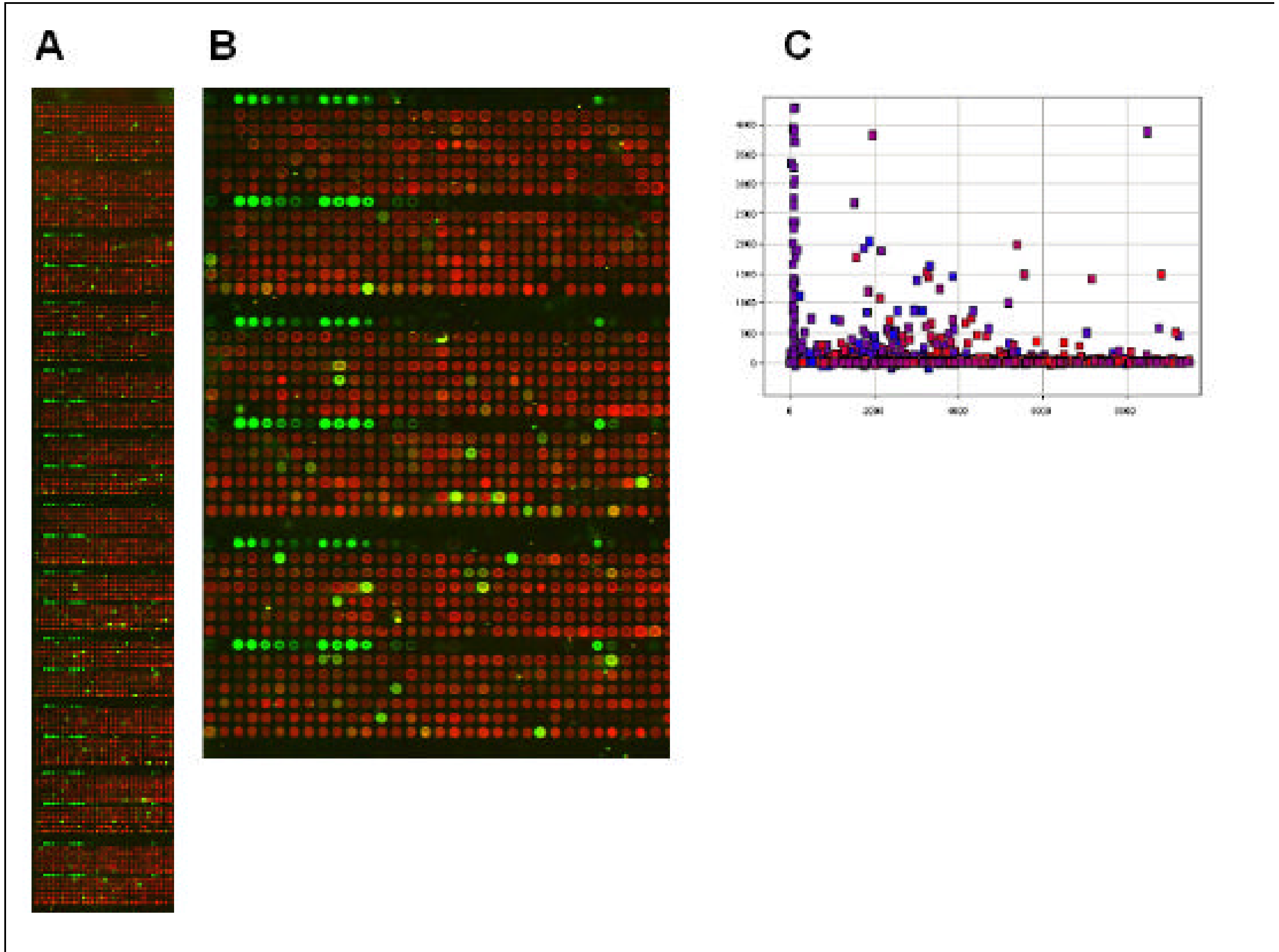

Figure 4

Automated microarray hybridization.

Comparison of a *Drosophila melanogaster* microarray hybridized with fluorescently labeled cDNA targets using **(A)** conventional manual hybridization and using **(B)** the AP Biotech automated slide processor

(ASP). The microarrays were hybridized with cye3 labeled adult cDNA and cye 5 labeled larval cDNA targets