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Integration of image analysis and robotics into a fully automated colony picking and plate handling system

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ABSTRACT

We describe here the integration of image analysis and robotics to produce a fully automated colony picking/plate handling system. Biological tests were performed to verify its performance in terms of sterilisation and accuracy of picking. The machine was then used by a single operative to pick a 36,000 clone cDNA library in ≈42 hrs over 5 days.

INTRODUCTION

The two key goals of the human genome project are the creation of a physical map of the genome and the determination of the DNA sequence [1,2]. Without the use of automation these goals will take an extremely long time to fulfil. Methods are therefore required that can be used in conjunction with automated instrumentation. The downstream side of the process has been developed with the use of a number of automated gel running machines (reviewed in 3) and there have been a number of developments in the preparation of template DNA for sequencing reactions [3–7]. The use of large clone libraries is central to the development of contig mapping. The main resource for physical mapping is a library of cloned DNA in one of a number of vector systems. The library is then screened with probes from a particular region. This can either be done using randomly distributed clones [8,9] or in an ordered arrayed of clones transferred to a membrane for screening by hybridization [10,11]. The former was a milestone in molecular biology and has been very successful in isolating and cloning genes from both genomic and cDNA libraries, however it has limitations if a particular library has to be screened many times, as the primary plates once made must be stored, while the screening takes place. This inevitably leads to contamination of the plates and eventually the library has to be re-plated and new hybridization membranes made. If the library is only available in limited quantities, as is the case for most primary cDNA libraries, then the library will need to be either re-made or amplified with a consequent reduction in representation.

In an arrayed library each clone must be picked into the well of a microtitre plate for both growth and storage. This is currently done by hand. The inoculated microtitre plates are then used to make high density membranes for screening by hybridization. The advantage of this system is that the primary library is archived and acts as a reference set. It can be indefinitely maintained by sub-culturing, as individual clones in microtitre plates, thereby avoiding under representation of clones. This system has the serious disadvantage in that each clone must be picked by hand. The best picking rate is 3000 colonies/day [10] with 1000/day being more achievable given human limitations. Thus a 5×10^4 clone YAC library would take 17–50 man days, or a 1×10^6 clone cDNA library would take 333–1000 man days. The time constraints and its tedious nature make the hand picking and gridding of libraries at present impractical although it is recognised that gridded arrayed libraries are a desirable resource for the long term screening, storage and cross referencing for physical mapping. The YAC cloning system [12] has been used to generate large insert clone libraries in yeast but as the transformation system relies on the regeneration of yeast spheroplasts in top-agar each colony must be hand picked. The scientific value of the YAC libraries for mapping purposes has obligated the picking of the libraries by hand which now serve as reference sets [11–18]. However it is not certain how widespread the YAC technology will become as a major barrier to entry is the the task of colony picking. The ability to pick colonies in a truly automatic mode would therefore aid the production of gridded arrayed libraries for screening purposes and allow greater access to mapping systems that have up until now been difficult to reach.

We describe here the integration of vision and robotics to produce a fully automated colony picking/plate handling system and the biological tests that were used to verify its performance. A 36,000 clone cDNA library was picked in ≈42 hours of machine time with the operator being required in attendance for 6 twenty minute change over periods.

MATERIALS AND METHODS

Images were obtained through a colour camera (TMC-516, Pulnix Europe Ltd, Basingstoke, Hants, U.K.) with a Cosmicar 12mm, F1.2 lens. The images were captured and stored on a frame digitising and storage card (DT2255, Data Translation, Marlboro,

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MA, USA)—digitises to 512×768 array of 8 bit pixels and finally displayed and processed on a MacintoshII fx™ computer system with an Apple 13", colour monitor. All image processing software was specifically written for this application by Visionways (32 Hiltom Rd, Ringwood, Hants, U.K.).

The main moving slides were two linear motors and drive systems, 200μm resolution, ± 20μm accuracy (Linear Technology Ltd., Rayleigh, Essex, U.K.). Microtitre plates were filled by means of a pump unit and 8 way manifold filling head (Wellfill 3, Denley Instruments Ltd., Billigshurst, Sussex, U.K.). The media bottle was pressurised through a sterile HEPA filter (060-33uu, M.G. Medical, Colchester, Essex, U.K.). The entire unit was supplied with clean air via an inline HEPA filter unit (MC Air Filtration Ltd., Gilligham, Kent, U.K.). Electronics were purpose built to drive standard stepper motors and d.c. motors. All other mechanical components were purpose built as detailed in the text and run from purpose developed machine movement software written in Pascal using THINK Pascal (Symantec, Cupertino, CA, USA). A standard Macintosh user interface allows the operator to easily change machine operating parameters.

Preparation of strains

Colonies or phage were grown on 8×12cm inoculum plates (Dynatech Laboratories Ltd., Billingshurst, Sussex). Each plate contained 50ml of agar and was allowed to set on a level surface. Colonies of *E.coli* were picked into 96 well microtitre plates (Falcon 3072, Becton Dickinson and Co., NJ, USA.). All media is of standard composition as previously described [19].

For *E.coli* sterilisation tests a pBluescript KS$^+$ clone in DH5α called FB3 was used. For yeast sterilisation tests AB1380 was used imbedded in top agar which closely mimics the appearence of yeast colonies after transformation with YACs [12]. For phage λ sterilisation tests the host was NM514 for an amplified cDNA library in λgt10. A lawn of *E.coli* cells or a lysis of phage λ was prpared by standard methods[19].

A total human foetal brain cDNA library in pBluescript KS$^+$ as ligation mix was a gift from Sabastian Meier-Everett at ICRF, London. The library was electroporated into electro-compedent host strain XL1-blue as described by the manufacturer (BioRad GenePulser). For the density tests 10μl, 25μl,50μl and 75μl of transformed cells were plated in 10ml of H-top agar. Once the agar had set a further 5ml of H-top was poured on to the plates. This was to ensure that all colonies grew in the top agar giving a uniform colony size. 5μl of ligation mix gave ≈40,000 primary cDNA clones, plated at a density of 300−500 colonies/plate on 2×TY+50μg/ml ampicillin. Colonies were picked into 125μl of 2×TY+50μg/ml ampicillin+1× freezing medium[10]. This allows the colonies to be grown in media and then transfered directly to storage at −70°C.

PCR screening for double picking events

PCR reactions were set up in 96 well PCR microtitre plates with each PCR reaction in a final volume of 10μl containing 1μl 10× PCR buffer (500mM KCl, 100mM Tris-HCl pH8.4, 15mM $MgCl_2$, 1mg/ml gelatin), 1μl of 2mM dNTPs, 1μl of 10μM T7 primer, 1μl of 10μM T3 primer, 0.1μl Cetus Taq Polymerase and 5.9μl water. From a microtitre plate containing the clones to be amplified all 96 clones were inoculated into the PCR microtitre plate by means of a 96 pin head and then 40μl of mineral oil was added. The PCR conditions were 95°C, 0.5min; 53°C, 0.5min; 72°C, 1min for 35 cycles followed by 72°C for 5min. The entire contents of the PCR were then run on a 1.2% agarose gel (data not shown) and the numbers of double bands observed.

DESIGN AND LAYOUT

The machine consists of two linear slides in the X,Y direction, a Z direction picker mechanism and a plate handling/storage system (figure 1a−c). The microtitre plates and the petri plates are mounted on a carriage on the Y linear slide , while the CCD camera and the picking head are mounted above on the X linear slide. The picker head consists of a six way picker needle on a wheel driven by a small stepper motor (figure 1b). In line with the X-axis is a sterilisation station. The sterilisation station consists of a 70% ethanol bath with a reservoir and adjacent heating element. The bath has two rows of brushes mounted just below the surface such that the needles travel through them. The input and output plates are housed in two carousel stacks with loading and lid lifting mechanisms for each and a microtitre well filling mechanism. The entire unit is housed within a steel framed box with a forced air HEPA filter unit providing a sterile atmosphere.

The machine is entirely controlled through a Macintosh fx. Images of the plates are captured through a CCD camera. Image analysis software running on the Macintosh fx automatically determines colony positions. The co-ordinates of the colonies in pixels are converted into machine co-ordinates which are then passed on to each of the actuators to perform the picking task.

OPERATION

Start Up

At start up the empty microtitre plates with lids on are loaded into the output racks which hold 52 plates in 4 racks of 13 plates. The plates containing colonies to be picked are loaded into the input side which holds 26 plates in 2 racks of 13 plates. The sterile media is then loaded. The user then sets operating parameters (number of plates, well fill volume) and image processing parameters using the Macintosh user interface program.

Picking

A flow diagram of machine operation is shown in figure 2. Solid lines show the logical flow of operations and dotted lines show information flow from the start up parameters. An analysis of the times for each event during the picking operation is given in table 1.

Microtitre and petri plates are loaded on to the machine by dragging the plate out from the shelf of a rack using a hooked arm and locating to a dead stop on the Y slide carriage. The lids are then lifted from the plates using a vacuum sucker pad. The racks can be rotated around the carousel to index a different rack into position. The vertical position of the rack can be varied to provide access to any of the 13 shelves. The microtitre wells are filled eight at a time.

The X and Y slides then move to position the petri dish beneath the camera for 6 images, in order to provide higher resolution. The colony positions within each image are then determined automatically according to the image processing parameters derived from the set up routine. These pixel co-ordinates are then converted to robot co-ordinates.

The X and Y slides are then moved to position the petri dish below the first needle of the picking head. The picking needle is moved down into the colony by actuating the Z motor, thereby

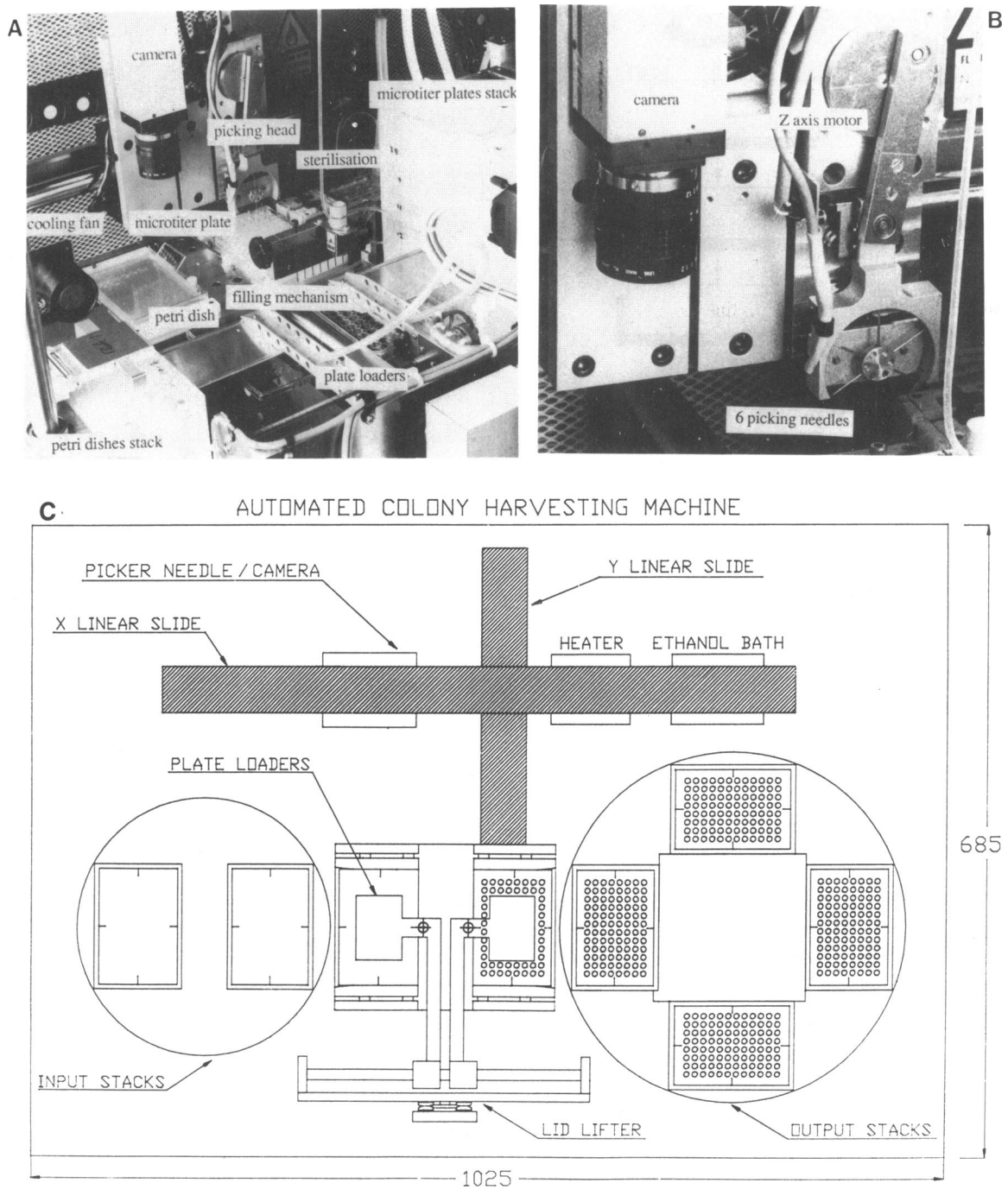

Figure 1. Plan and details of the automated colony picker. A) view of the main working components of the system showing their relative positions. B) close up view of the camera and z axis with 6 way picker needles. C) drawing of a plan view of the machine giving overall dimensions and layout.

transferring some of the colony cells on to the needle tip. The needle is then withdrawn from the petri dish. A new, sterile needle is then selected by rotating the picking needles through 60° and the picking procedure is repeated for each needle. A similar procedure is then carried out to inoculate the growing medium in each of the microtitre plate wells with cells from one of the needles.

The needles are then sterilized according to the sterilization routine selected by the user as determined by the colony type. The whole procedure is repeated, unloading processed plates and loading new plates as required, until either the supply of microtitre plates or the supply of petri plates is exhausted.

A new run can be set up by replacing the microtitre plates and petri dishes with unprocessed ones and renewing the stock of growing media in around 20 minutes.

Image analysis

The petri dishes are viewed using a 'dark field' lighting arrangement in which the plates are illuminated obliquely against a matt black background. This considerably enhances the contrast

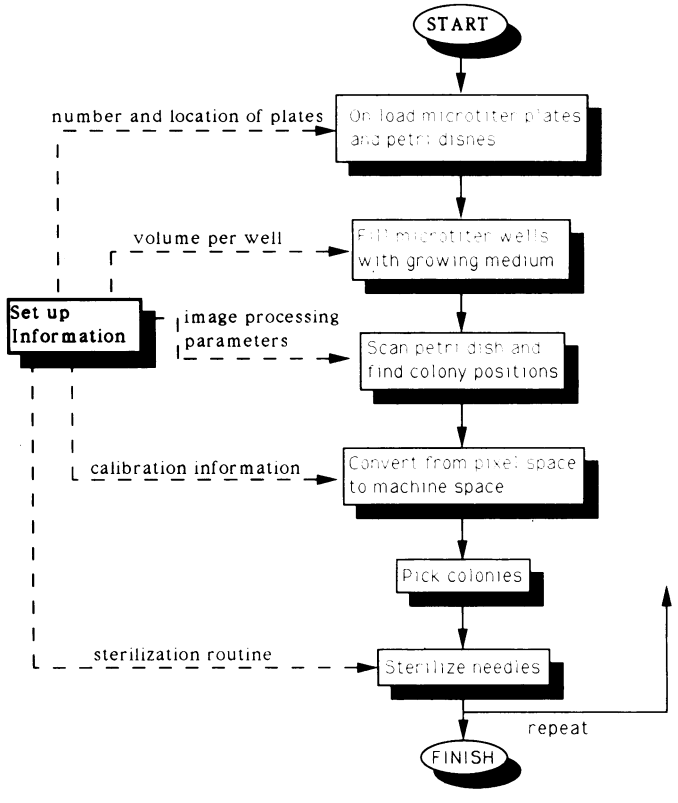

Figure 2. Flow diagram of machine operations. Solid lines show the logical flow of operations and dotted lines show information flow from the start up parameters.

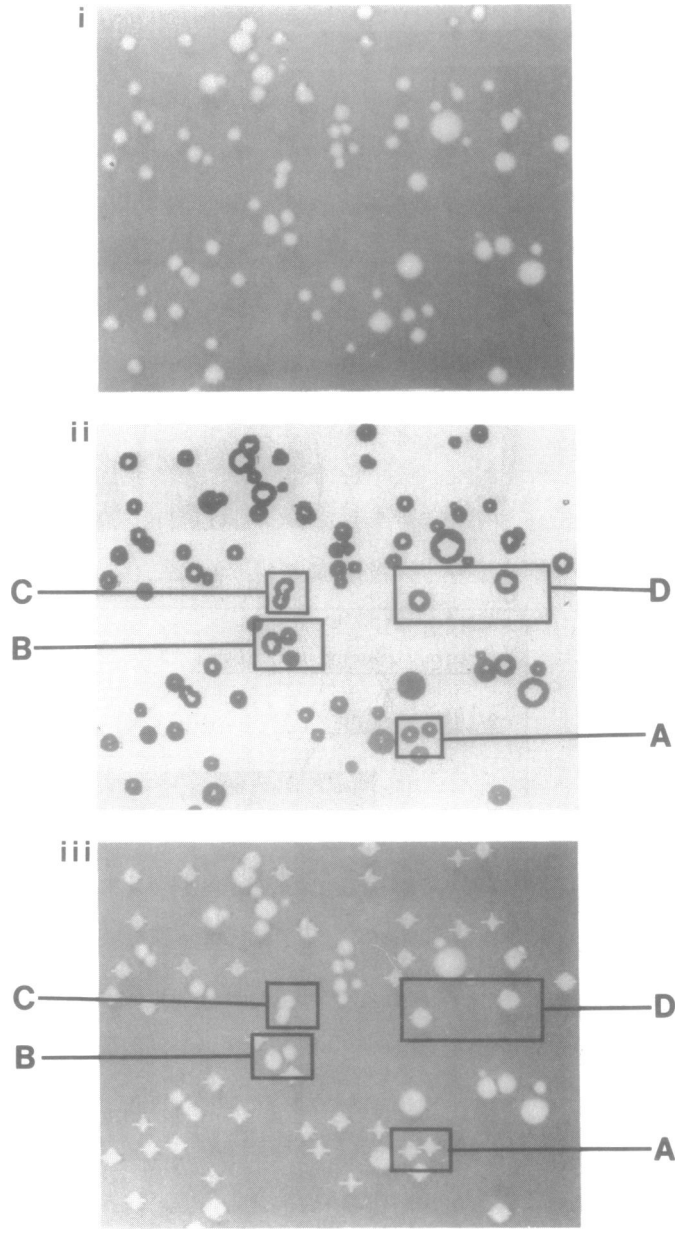

Figure 3. Three images of 1/6 of a plate of *E.coli* colonies illustrating the stages of image processing taken directly from the monitor on the Macintosh fx. i) 5 frames averaged together to reduce noise effects. ii) local thresholding of the image. Where colonies are far apart (as at D) discrete hoops are present. Where colonies are very close together (as at B) or have actually merged (as at C) these hoops join to form figures of eight. At A the colonies were not close enough to cause the hoops to join. iii) The final operation is to reject objects that do not pass size, shape, and grey level criteria. Those objects that are to be picked have a cross placed on them. The pixel co-ordinates are then converted to machine co-ordinates and the objects picked.

between phage colonies and background and eliminates shadowing problems that occur when reflective lighting from above is used.

Finding colonies within an image is a three stage process involving digitising an image, finding all objects within the image and using measurements of each particular object to distinguish whether it is a discrete colony or some other object. This process is carried out fully automatically.

Firstly 5 frames of video at one of the 6 positions are digitised. These frames are averaged together to reduce noise effects. An image of an *E.coli* plate is shown in figure 3a.

The second stage is done using local thresholding, a morphological edge detection algorithm. Local thresholding finds the maximum difference in grey level amongst the pixels surrounding a central pixel; this window can therefore be 3×3, 5×5, 7×7 pixels. If the maximum grey level difference is greater than a given threshold value ($0-256$) then the central pixel is set to black, otherwise the central pixel is set to white. The result of this operation on the initial image is shown in figure 3b It can be seen that where colonies are far apart (as at D) discrete hoops are present. Where colonies are very close together (as at B) or have actually merged (as at C) these hoops join to form figures of eight. At A the colonies were not close enough to cause the hoops to join.

The final operation is to reject objects that do not pass size, shape, and grey level criteria. If the object area is not within a minimum and maximum area range set by the user during the set up stage then it is rejected. This removes any small particles of dust, faint satellite colonies or large areas caused by agar imperfections. Next the maximum and minimum values of the radius of the objects are found from a polar plot of the edge of the object. If this difference is greater than a value chosen by the user during set up then the colony is rejected. For the *E.coli* plate this value was 20% of the average radius value. This checks colonies for circularity and rejects colonies that have merged. Finally the centre grey value is compared with the background

Table 1. Cycle time analysis for picker operations

Operation	Processing Time (s)	Number of colonies processed	Time/colony(s)
Load microtitre plate	84	96	0.87
Fill microtitre plate	12	96	0.12
Load petri plate	84	400	0.21
Image analysis	17	67	0.25
Picking	4	6	0.67
Inoculation	3	6	0.50
Sterilisation*	5	6	0.83
Set up	1200	4992	0.24
TOTAL TIME/COLONY			3.63

* Given time for *E.coli* or yeast colonies, for phage plaques there is an additional sterilisation time of 1.2s/colony for heat sterilisation.

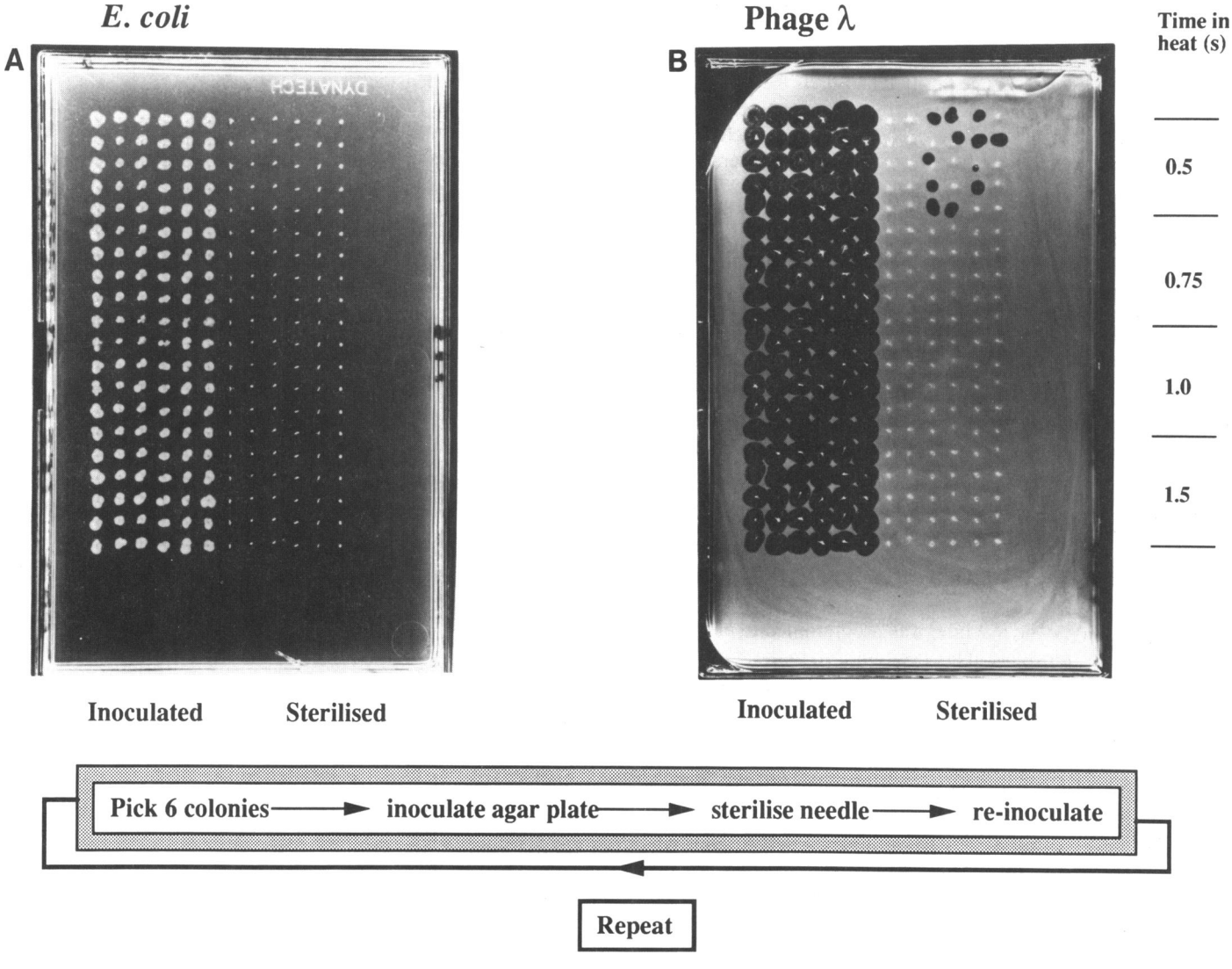

Figure 4. Illustration of the effectiveness of sterilisation for *E.coli* and phage λ. The robot was programmed to pick from a lawn of cells or confluent phage, inoculate at six positions, go to the sterilisation station, sterilise the needles, return to the inoculated plate and re-inoculate the plate at a further six positions. This procedure was repeated 20 times. The plates were then incubated overnight and observed. a) shows the results using *E.coli* . The needles were sterilised by spinning in 70% ethanol followed by a brief spin through the heating element to remove excess ethanol before picking the next six *E.coli* . b) shows the results with phage λ. The needles were sterilised by holding in the heating element for 0.5−1.5s. After heating the needles were spun in the 70% ethanol bath to cool the needles, before picking the next six phage. Phage were picked into a lawn of NM514.

surrounding the object. If this difference is within user-defined limits (> +40 for the *E.coli* plate) then the object is passed as a colony and a cross is placed on the colonies which are to be picked (figure 3c). This algorithm discriminates between blue and white colonies for recombinant/non-recombinant selection in *E.coli*, phage M13 by gray scale alone, without the need to resort to colour image analysis.

Automatic optimization of local threshold differences is carried by varying the difference and finding the maximum number of valid colonies. The local threshold differences at which the maximum occurred is then used for all subsequent plates.

Conversion to machine co-ordinates

During set up a plate is put on to the Y slide of the machine that has accurately placed dots in a grid pattern with 2mm spacing between each dot. The height of this plate is set by the operator such that the plane of the grid is at the same height as the plane in which the colonies or plaques are growing. This is assumed to be constant for a given batch of plates as each plate is poured level with the same volume of agar. The robot then positions the camera above the test plate position and finds the co-ordinates in pixel space of each of the holes using a similar process to that outlined above for finding colonies. The positions of each of these grid holes are stored so that, during machine operation, a coordinate in pixel space can be transformed to a coordinate in robot space, taking into account agar height, spherical aberrations, and lens imperfections.

To accurately ascertain the position of each picking needle the robot punches six holes per needle in a blank piece of paper. The positions of these holes in robot space are found using the procedures outlined above for finding colonies. The average offset from the nominal needle position is calculated for each needle and is automatically corrected for during machine operation. The position of the needles can vary due to manufacturing differences or when the needles are removed for cleaning as is periodically necessary.

BIOLOGICAL TESTS

A number of tests were performed to assess the accuracy of picking and efficiency of sterilisation.

For each cell type the machine was programmed to pick from the lawn of cells, inoculate at six positions, go to the sterilisation station, sterilise the needles, return to the inoculated plate and re-inoculate the plate at a further six positions. This procedure was used with various sterilisation regimes. In the case of phage λ, phage were picked from a confluent lysis plate directly into a lawn of NM514.

E.coli and Yeast

For yeast and *E.coli* the most efficient form of sterilisation was to spin the needles in the 70% ethanol bath (figure 4a, data for yeast not shown). This proved to be very effective at killing all cells remaining on the needle after initial inoculation. Any residual ethanol was evaporated away by briefly stepping the needles through the heating element.

Effect of heating and cooling upon phage sterilisation

Attempts to use liquid sterilisation either 1% Clearsol or PreSept (1 tablet in 250ml water gives ≈5000ppm chlorine) for phage were ineffective (results not shown) therefore a heating element was incorporated into the machine next to the 70% ethanol bath. This gave a temperature of ca 350−400°C in the void around the element.

The optium heating time for killing phage was determined by holding the needles in the heating element for between 0.5−1.5 seconds. It was found that if excess heat was used then the needles became hot resulting in loss of plaque formation (results not shown). The needles were cooled by spinning in 70% ethanol. As shown in figure 4b all phage were killed by heating the needles for times greater than 0.75s.

Accuracy of picking individual colonies

The accuracy of the colony picking machine is determined by a number of factors including positional accuracy of the mechanism i.e. two linear slides, the up down picker head as well as the accurate conversion of image co-ordinates into machine co-ordinates. The overall positional accuracy has been determined as ±0.3mm. This is sufficient accuracy to be able to find and pick colonies as small as 0.5mm in diameter. The possible errors that might occur were tested on the ability to pick colonies from a foetal brain cDNA library in *E.coli* that were plated at various densities.

The total human foetal brain cDNA library was plated out as described in methods. Four aliquots gave a good range of cell densities of 4, 8, 16 and 32 colonies/cm.sq. Three plates at each density were prepared and loaded into the colony picking machine and picked into microtitre plates. From each batch of microtitre plates 96 colonies choosen randomly were used in a PCR reaction

Table 2. Results of accuracy of picking at various densities.

Density/cm.sq	Number Colonies/plate	Number Double PCR events/96 clones*
4	356	2
8	720	4
16	1496	3
32	3051	2

* 98% of the colonies that grew gave a positive PCR product.
Colonies were picked from four different densities plates. The presence of an insert from each of 96 cDNA colonies, for each density was checked by PCR as described in materials and methods. The number of double PCR products were recorded.

Table 3. Results of foetal brain cDNA library picking run.

Parameter	Number
Number of microtitre plates	375
Number of colony plates	100
Average number of colonies picked/plate	360
Average number of colonies left/plate⁺	1−20%*
% colonies growing after picking	97.3%
Number of runs	7.2
Turn around time between runs	20min
Total machine picking time	42.2hrs
Number of colonies picked	36,000

* The number of colonies left was highest if the colonies were unevenly spread over the plate.
⁺ Colonies that did not meet image processing criteria.
Image processing:
Minimum colony area : 15 pixels
Maximum colony area : 300 pixels
Local threshold difference : 20−30
Local threshold window : 3×3 pixels
Maximum radius difference : 3 pixels

to amplify the insert as described in materials and methods. The numbers of double PCR products was determined (Table 2).

Each individual cDNA clone picked should have given rise to a single PCR product. If more than one product was present then a double picking event could have taken place. The source of this error could have come from three sources: image analysis, mechanical or biological. This experiment demonstrated the level of double picking events for each density of plate. As can be seen from table 2 there is no appreciable difference in the number of double picks for the different densities. The increase in colony density also gives rise to a concurrent increase in the numbers of satellite colonies. Most of these are not recognised by the image processing as they are not as dense as genuine colonies. The optimum density at which to pick colonies was therefore determined by biological constraints.

From the results of the above experiment the total human foetal brain cDNA library was plated at 300−500 colonies per plate (4−6 colonies/cm.sq). The plates were then loaded into the machine and over a period of 5 days 36,000 colonies were picked. The results of this large scale picking are given in table 3.

Reverse picking and plate tracking

An important task down stream of the colony picking process is the production of high density filters to be used for screening[10,11]. The colony picking machine is able to perform this task by placing the picked microtitre plates in the output carousel and putting plates with membranes in the input carousel. The machine then reverse picks from the microtitre plates onto the membranes. The density at which the colonies are reversed picked is predetermined by the user as either 5, 9, 16, or 25 times 96 in the space of one. Once the machine has finished picking then the membranes are placed onto agar and the colonies grown overnight. The DNA from each colony is then fixed to the membrane by conventional means [19]. This process is not as fast as other purpose built high density spotting devices [20,21] because the colonies are reversed picked in multiples of 6 instead of 96, however the entire process up to growing the cells is entirely automated and unmanned. In order to help in the indentification of individual microtitre plates character recognition software was written that is able to read a 7 digit number of the side of the microtitre plate via a 45° mirror. This is the first stage in the development of a data handling and retrival system for colonies. The other half of this process is the identification of hybridization signals and relating this back to the appropiate microtitre plate for the retrival of the correct clone. This task has been achieved by use of a sonic digitiser and custom software written for a Macintosh (Jones and Davies, unpublished).

DISCUSSION

The task of colony picking represents a major bottle neck in the production of gridded arrayed libraries, and is also important in large scale screening for biologically activity in the food and pharmaceutical industry. The gridded arrayed libraries are an important resource for mapping and consequent sequencing projects of the Human Genome Programme. The system we chose to develop integrates all the elements that are required for unmanned operation. The system is a stand alone unit that requires no help from the operator once the pre-run set up procedures are completed.

Further work is being carried out on the colony identification procedure to take account of agar height differences within a batch of petri plates which can vary either due to aberrantly poured plates or evaporation. Therefore three dimensional imaging techniques such as stereo imaging are being investigated that automatically calculate the distance of each object from the camera plane. Methods of carrying out the image processing as a background task, while picking is being carried out, are also being investigated.

The sterilisation tests demonstrated that spinning the needles in 70% ethanol was adequate in killing yeast and *E.coli* colonies. This regime was used in picking the cDNA library without cross contamination problems.

The only method that effectively killed phage was to heat the needles in a heating element and cool by spinning in 70% ethanol.

The accuracy of picking at various densities was determined by the number of double picking events. There was no significant increase in double picking events for the higher density plates, verifying the picking accuracy.

The library picking demonstrated the ease of use of the machine and the ability to work unmanned. The percentage of colonies that did not grow in the microtitre plates after inoculation was 2.7%. This could be due to non-viable colonies or a missed pick either due to mechanical or image analysis error. This level compares well with manual methods and is quite acceptable for most purposes.

The picked libraries can also be reversed picked by the machine on to high density 8×12cm filters at either 5, 9, 16 or 25 times density.

A MkII machine is under test which improves the efficiency by having a different physical layout and increases the speed. A new picking head has been designed that allows parallel inoculation and parallel sterilisation of the picker needles thereby improving cycle times.

The machine described here and the MkII will be of enormous use in generating large gridded array libraries. It exceeds the performance of previously described machines [22−24] and operates unmanned. The improvements in time and accuracy afforded by the machine described here will make it possible to consider the picking and gridding of most randomly arrayed libraries. The removal of this significant bottle neck in genome analysis should make it possible to study primary cDNA libraries, YAC libraries and allow wider access to resources that are currently restricted by the technology.

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