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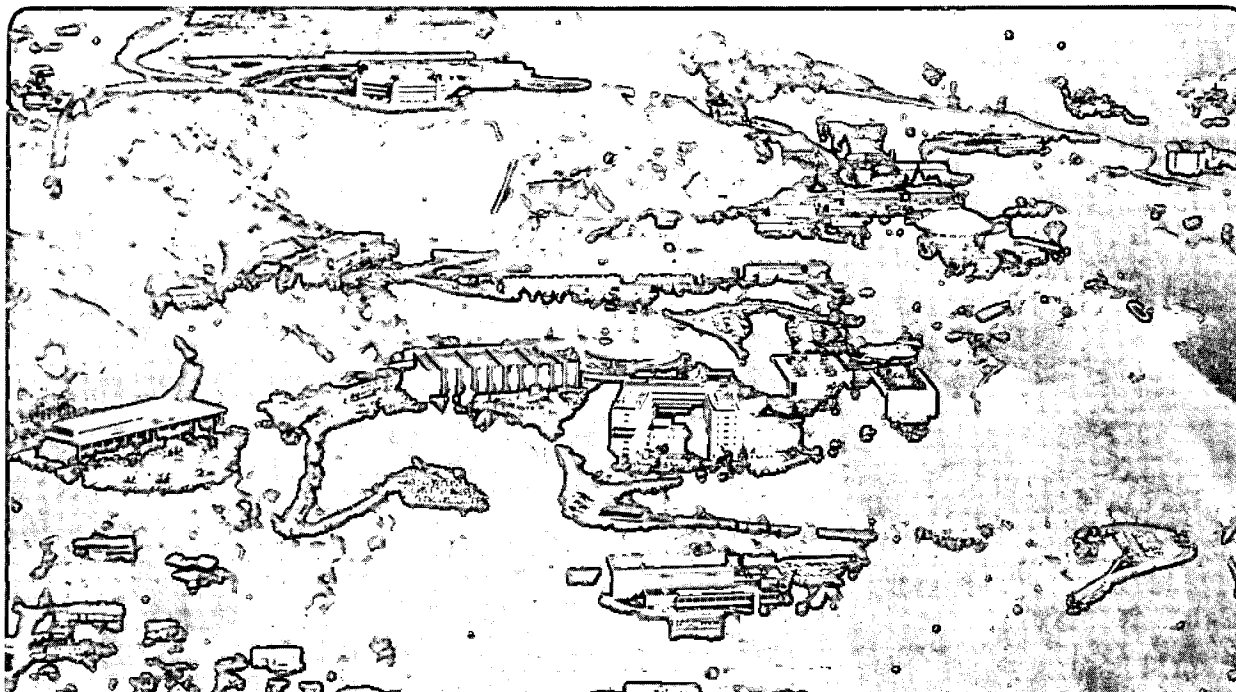# Lawrence Berkeley Laboratory
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ABSTRACT

We describe a system which applies image processing and robotic techniques to automatically pick individual colonies from square Petri dishes and array them in 96 well microtiter plates. Digital images of the colony distribution in the dishes are acquired using a video camera and frame buffer. Commercial image processing software is used to identify individual colonies and determine their locations. A Hewlett-Packard Microassay System robot reads the resulting coordinate file for each dish, picks cells from each identified colony and transfers the cells into a microtiter plate well. A disposable pipet tip is used as the sterile implement for colony picking. Custom holders position the dishes accurately and provide common coordinate systems for imaging and picking. The system is calibrated to account for the depth of agar in the dishes. The robot can process up to 10 dishes and 20 plates (1920 colonies) in a single run. It has successfully arrayed a cosmid library of the *S. pombe* genome consisting of approximately 6000 clones in 30 Petri dishes, in about 40 hours of robot time. Future enhancements to the system are discussed.

INTRODUCTION

A major emphasis of the Human Genome Project in its early phases is the construction of physical maps of individual chromosomes. Many diverse elements must come together to accomplish this important task including the large scale production of selected DNA fragments required to produce the data for map assembly. In particular, large clone libraries of various types and with different vectors are essential to the process, regardless of the mapping strategy chosen.

The production of these libraries involves a number of steps, several of which are repetitious and require a minimum of scientific judgement. Consequently, they lend themselves to automation. After clones are created and grown up as colonies in a growth medium, they are *picked, arrayed, screened* for purity, *hybridized* with a variety of probes, *replicated* (if the library promises to be valuable), and *prepared for storage* until needed. At our laboratory, each of these steps has either been automated or is currently undergoing that conversion. In the simplest case, this may involve simply the mechanical transfer of clones from one container to another (although even this step is not entirely trivial when dealing with biological materials). Most of these applications, however, require the development of peripheral devices which help to specialize the robot to the particular task.

This paper focuses on the integration of an image processing system with a commercial robot for the purpose of picking bacteria or yeast colonies from Petri dishes and arraying them in 96 well microtiter plates, prior to further processing. While we have not attempted to physically integrate the two units, nor does that seem necessary at this point, this is nevertheless an initial attempt to use machine vision in a practical way in a molecular biology laboratory. However, an important factor in the success of the procedure has been the ability to design and construct specialized hardware which is used in conjunction with the existing robot to address specific tasks.

In principle, the ideas behind this procedure are simple (Figure 1). The robot, a commercially available device originally targeted for the pharmaceutical industry, is able to move a mechanical hand to a given position in cylindrical coordinate space. Standard image processing software is capable of locating the individual clone colonies (either yeast or bacteria) and determining their locations relative to a coordinate system local to the Petri dish. This set of coordinates (or, in general, several such sets at a time) can then be transferred to the robot's computer controller as the critical input to the process. The robot can then pick the colonies individually and transfer them to the microtiter plates. The process by which this conceptual idea is transformed into a system that actually works in the real world is explained in the following sections.

METHODS AND MATERIALS

We plate out the library to be arrayed in plain square 100 x 100 x 15 mm Petri dishes (Nunc Lab-Tek 4021). Square dishes are preferred to circular in this application since they greatly reduce the possibility of misorientation. These dishes have no markings to interfere with imaging, and the edges allow accurate alignment in custom-built holders during both imaging and picking. To ensure uniform results, we fill all dishes with agar to the same level and keep the lids on except during actual image acquisition and colony picking.

Imaging System

The imaging is done with a charged coupled device (CCD) video camera (model 6500, Cohu, Inc., San Diego, CA) interfaced to a frame buffer board (model MVP-AT, Matrox Electronic Systems, Dorval, Quebec) operating in an IBM AT personal

computer. Each dish to be imaged is loaded manually into a custom acrylic plastic holder (Figure 2) where it is firmly registered by a spring-loaded clip. A cutout in the holder beneath the dish allows illumination from below by a fluorescent light table on which the holder sits. The camera views the dish and holder from above through a Nikon Nikkor 28 mm f/2.8 lens positioned about 0.5 m above the dish, giving a field of view approximately 128 x 96 mm. We adjust the camera aperture and the analog gain and offset controls to enhance the contrast between the colonies and the background.

The field of view in the acquired image is adjusted to include three reference dots on the holder beside the dish. These marks establish a Cartesian coordinate system in which the location of the dish is precisely known. Since the frame of reference is on the holder, the positioning of the holder under the camera is not critical, an important feature of our system.

Imaging Procedure

We employ two commercial software packages to do the image acquisition and processing. First, Image-Pro II (Media Cybernetics, Silver Spring, MD) is used to digitize and store in the frame buffer a 512 x 480 pixel 8 bit image of a dish. The detection and measurement of objects is then done with ImageMeasure/IP Counting and Sizing Module IM5100 (Microscience, Federal Way, WA). The user selects (using a mouse) the rectangular boundaries of small regions surrounding each of the three reference points, and a larger region on the dish enclosing as many colonies as can be unambiguously identified (Figure 3). Colonies on or outside the latter boundary are excluded from automatic processing but can later be picked by hand. A gray level detection threshold is also established by the user.

ImageMeasure/IP automatically identifies all objects darker than the threshold, and writes to a floppy disk file their centroid coordinates and areas (in pixels) as well as

their "roundness coefficients". These latter values are proportional to area divided by circumference squared (normalized to 1.0 for a perfect circle), and may be used to distinguish good colonies from overlaps and artifacts. Since the reference points establish the coordinate system for the entire dish, they must be at the beginning of the file.

A separate file is generated for each dish. The files for a library are given names consisting of a common root followed by a three digit sequence number, e.g. SPCOS001, SPCOS002, SPCOS003, etc. The dishes are similarly labelled. This scheme aids the robot in finding the files when processing a sequence of dishes.

Robotic System

The actual colony picking is performed using a Hewlett-Packard (Avondale, PA) Microassay System, a robotic system for automating chemical assays. In addition to a robotic arm, our system includes stackers, workstations, and interchangeable hands for manipulating standard microtiter plates and pipet tips, eight programmable syringes for pipetting and dispensing fluids, and a control computer with associated software. The arm is located in the middle of a 1 m by 2 m table and can reach the other hardware by moving through a cylindrical space whose dimensions are: height 0 to 347 mm, azimuth 0 to 360 degrees, and radius 235 to 555 mm. The gripper hand extends the radial reach to approximately 715 mm. Although Hewlett-Packard has not published data on precision, we have measured the repeatability of arm motion to be better than 0.5 mm at the station we use for colony picking.

Custom Hardware

The robotics system is readily adaptable to new applications, and we have developed specialized hardware and software specifically for colony picking. The most important new item is a robot workstation which grips a Petri dish in a manner identical to that used on the imaging system (Figure 4). After firmly attaching the workstation to the table, we "teach" the robot its position by manually moving the arm to two reference pins on the workstation. The robot is then capable of mapping any point in the Cartesian space of the dish into its own cylindrical coordinate system, using software described below.

We have chosen to use standard 200-microliter sterile plastic pipet tips as the actual picking tools, since the robot is already adapted to their use. To hold the tips as straight as possible we have built a special hand with a 40 mm long tapered shaft (Figure 4). Even using this hand, we see displacements at the pipet tips of up to 0.5 mm off axis, due to the curvature of the plastic. The sterile tips are loaded from racks of 96 and are disposed of after use.

Because of the lack of hardware designed for the 100 mm x 100 mm Petri dishes, we have built custom stackers for the dishes, and a workstation to hold a lid while its dish is being picked. We have expanded the robot's repertoire of actions by manually teaching it the proper motions for handling each new piece of hardware. These motions are stored on hard disk by the control computer, and are invoked from programs written in a language similar to BASIC.

Colony Picking Procedure

To pick the colonies for a number of Petri dishes, the user first loads them into a stacker in the same order and orientation as used during imaging, and loads the disk

of colony coordinate files into the floppy drive of the robot control computer. The picking program requests the number of dishes and the file name of the first dish. It then scans all the files to determine the total number of colonies, calculates the number of microtiter plates required, and requests that the user load the proper number of plates and pipet tip racks into their stackers. Up to 10 dishes and 20 plates may be processed in a single run. The plates have previously been filled with growth medium using another robotic procedure. The program asks for the starting well number for the first microtiter plate, in case that plate was partially used on a previous run. Finally, the program asks for the name of a calibration file, explained below.

The actual picking of colonies then begins. As the program calls for dishes, plates, and tip racks, the robot uses its standard gripper hand to move them from stackers to workstations and back. For each colony, the robot acquires a pipet tip from the rack by firmly inserting the shaft of the previously described custom hand. After picking the colony, the tip is scraped off into a disposal chute by drawing the shaft between the teeth of a fixed rake.

As previously mentioned, the first three points in each file are the pixel coordinates of the three reference marks on the dish holder used during imaging. Using these values and the known locations of the dish relative to the reference marks, the picking program computes X and Y scale factors and offsets for the dish, and also accounts for any rotation of the dish and holder on the light table. The program then converts the pixel coordinates of each colony into millimeters relative to the dish axes, and finally into cylindrical robot coordinates for the actual picking operation.

However, the fact that the colonies and reference marks are in different horizontal planes creates errors in the imaged colony locations (Figure 5A). These horizontal displacements amount to 0.9 mm for colonies most distant from the camera axis in dishes with 8 mm agar depth. We correct for this by means of a lucite calibration plate whose height we set equal to the height of the colonies above the reference marks on

the holder, and which contains precisely inscribed calibration points (Figure 5B). We image this plate and create a calibration file containing the pixel locations of the calibration points and reference marks. The picking program reads this file and adjusts the positions of the reference marks to appear as if they were in the same plane as the colonies, thus eliminating the errors.

The program is capable of rejecting objects failing to meet preset criteria of area and roundness. At present we reject only those objects exceeding 10 mm^2 in area, which are likely to be artifacts such as bubbles.

Colony picking proceeds unattended until the last dish is completed or the supply of plates and tips is exhausted. In the latter case the programs waits for new plates and tips to be loaded and then resumes. At its conclusion the program prints out the last well number used, so that the wells remaining on the last plate may be used on a subsequent run.

RESULTS

The system just described has arrayed a cosmid library of the *S. pombe* genome, plated out in 30 Petri dishes. The time to image the dishes was approximately 6 hours. In about 40 hours the robot picked approximately 6000 colonies, for a sustained rate of 150 colonies per hour. Most of this processing was done in unattended overnight runs of up to 12 hours. Approximately 1600 additional colonies, which were too close to the dish edges to be imaged, were picked by hand.

Typical diameters of the *S. pombe* colonies were 1 - 3 mm. The pipet tips left small craters where they hit the agar; these were around 1 mm in diameter. The maximum center-to-center errors seen between colonies and tip craters was also 1 mm. Most of the errors were 0.5 mm or less, so that complete misses were extremely rare. Of the wells filled by the robot, 99.5% grew cells.

DISCUSSION

The successful implementation of automated colony picking is an important step in facilitating the scale-up of biochemical procedures necessary for mapping and sequencing the human genome. The project required integration of data obtained from image processing with the mechanical variables required for robot operation, combined with the use of specialized mechanical hardware. This approach may serve as a model for automation of other biochemical procedures requiring visual observation and physical manipulation of objects. Examples of such procedures include automatic selection and removal of DNA bands from electrophoresis gels, and loading of inserts for pulse-field separations.

Our experience with this system has led to several ideas for enhancement. First, the spatial resolution of the imaging system could be improved if the camera and frame buffer were fully synchronized. By virtue of its sensor design, the CCD camera captures an image in discrete pixels. The data are then shifted out at a fixed rate and converted into an analog video signal, which is digitized and stored by the frame buffer at a rate asynchronous with the camera's shift clock. If the camera and buffer were synchronized so that their pixels exactly matched, the imaging resolution would be effectively doubled.

Second, the robot accuracy could be increased by correcting for the curvature of individual pipet tips. This could be done by using a translucent tip holder with a light source at the top of the shaft, so that the shaft and tip form a light pipe. After picking up a tip, the robot would position it to illuminate a four quadrant photodiode at a known location on its table. The differential signals from the opposing pairs of photodiode segments would be used to correct for the X and Y offsets of the tip from the true axis. We have done preliminary experiments which indicate this approach is feasible.

We are also considering alternatives to pipet tips as the picking implements. One option is to use a short length of stiff wire or plastic filament, which would be clipped off and replenished from a spool after picking each colony. Sterility, tip bending, and tool weight could present problems with this approach. Another option is to use a stainless steel pin and sterilize it with ethanol and electric heat after each use. However, the sterilization would increase the time to pick each colony from 25 to 40 seconds, according to experiments we have done.

Finally, we plan to use bar code labels on the Petri dishes and microtiter plates, and to equip the robot with a bar code reader, so that the dishes would be correctly identified no matter what their order in the stacker. This would ultimately allow us to integrate the imaging and robot control computers with a laboratory-wide database which would automatically track the clone libraries through all stages of their processing, including the use of other instruments such as DNA synthesizers and sequencers.

ACKNOWLEDGMENTS/DISCLAIMER

FIGURES

Figure 1   Basic components of the colony picking system.

Figure 2   A Petri dish held in place in the imaging holder by a spring-loaded clip at the right rear corner. The reference marks (dots on columns at the other three corners) provide a local coordinate system. The dish measures 100 mm x 100 mm. The colonies are clones in an *S. pombe* cosmid library.

Figure 3   Digital image of a Petri dish. The image processing software has drawn white outlines around the colonies it has detected in the rectangular window drawn by the user. The three reference marks, visible as dots next to the dish at upper left, lower left, and lower right, are detected in separate windows.

Figure 4   Robot picking a colony with a pipet tip on a hand specially designed to hold the tip straight. A spring-loaded clip (foreground) registers the Petri dish in a holder similar to the one used during imaging.

Figure 5A   Apparent location of a colony is displaced laterally outward, due to its height D above the reference marks.

Figure 5B   A calibration plate is used to make the reference marks appear as if they were in the plane of the colonies.
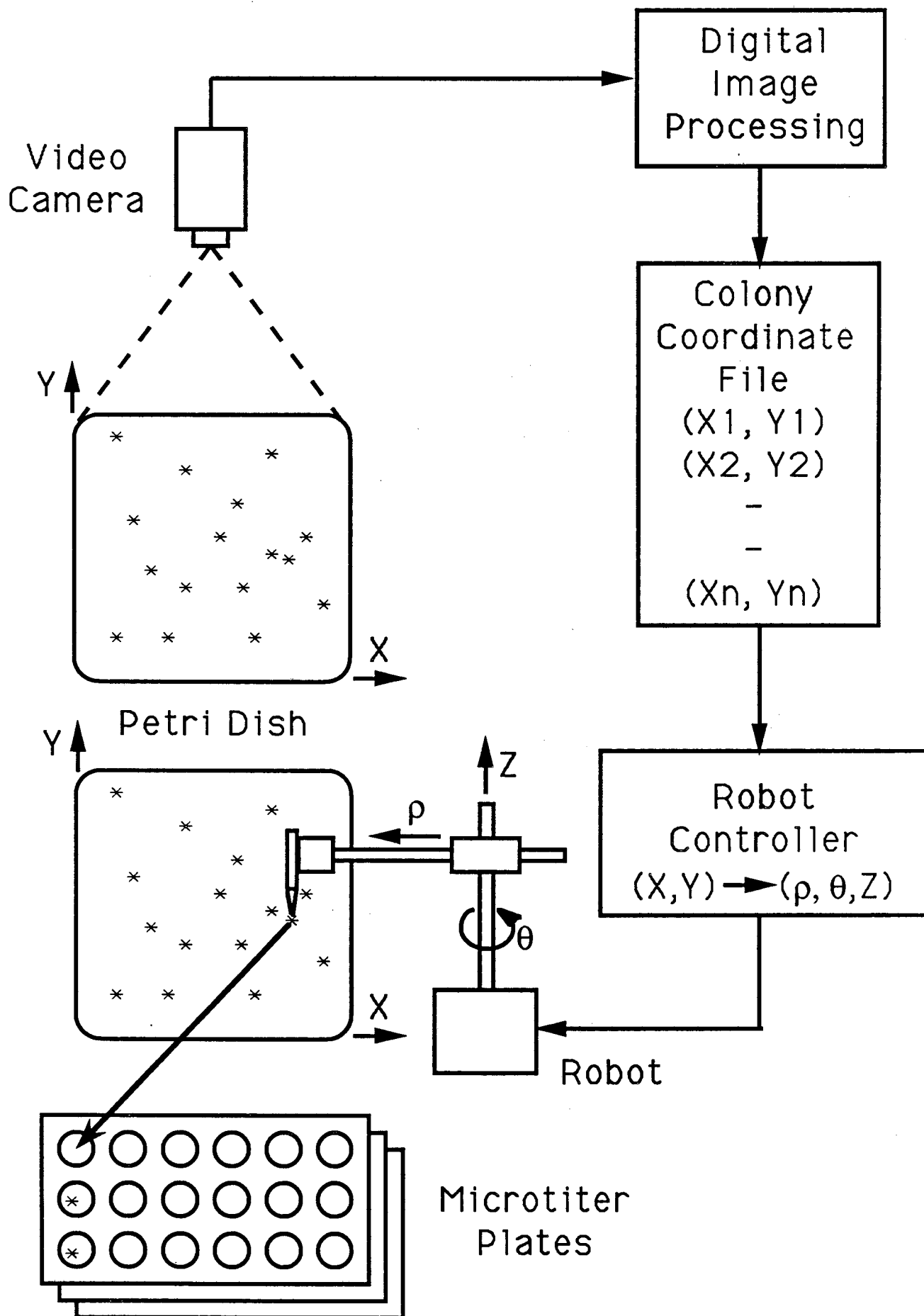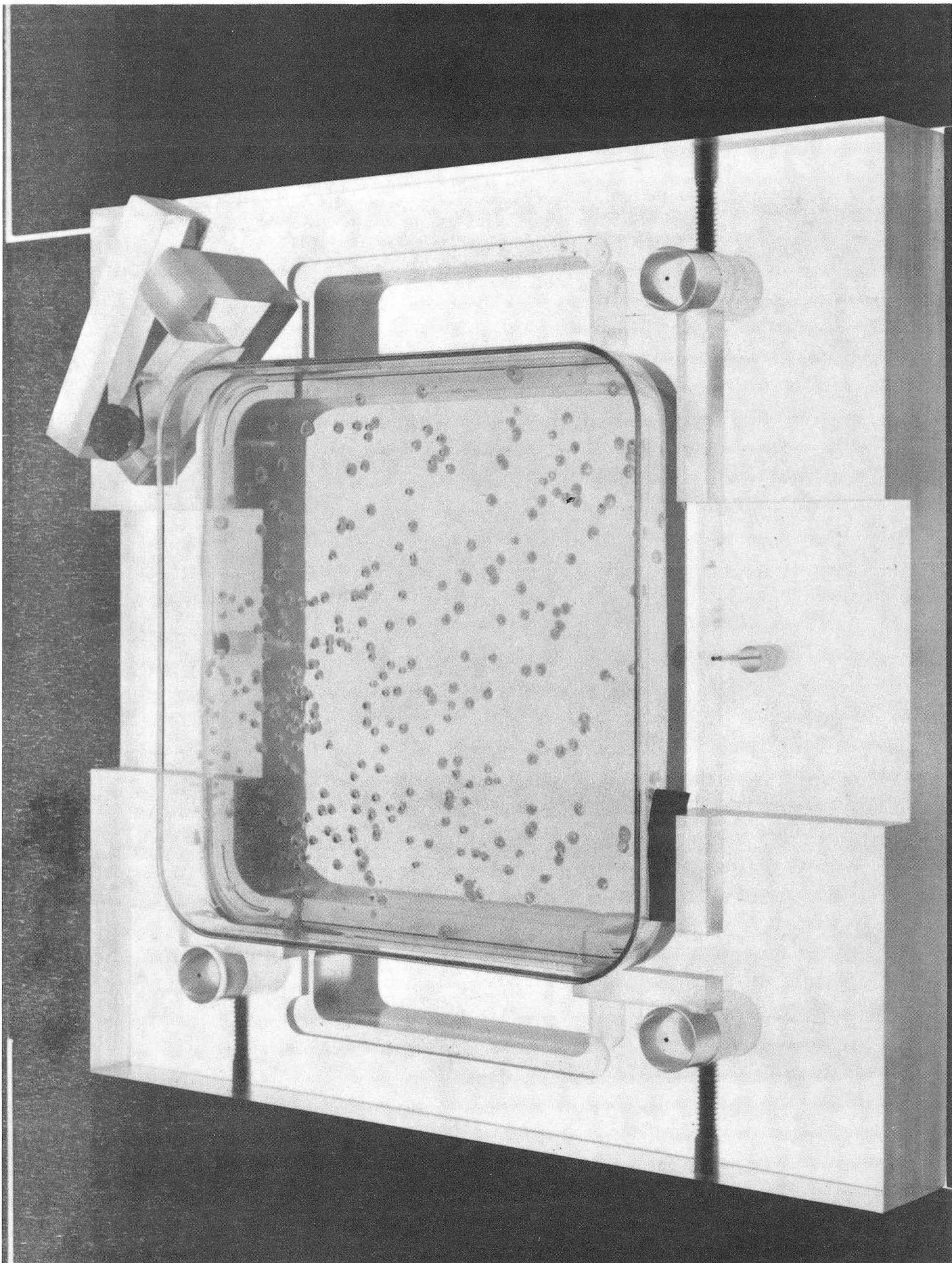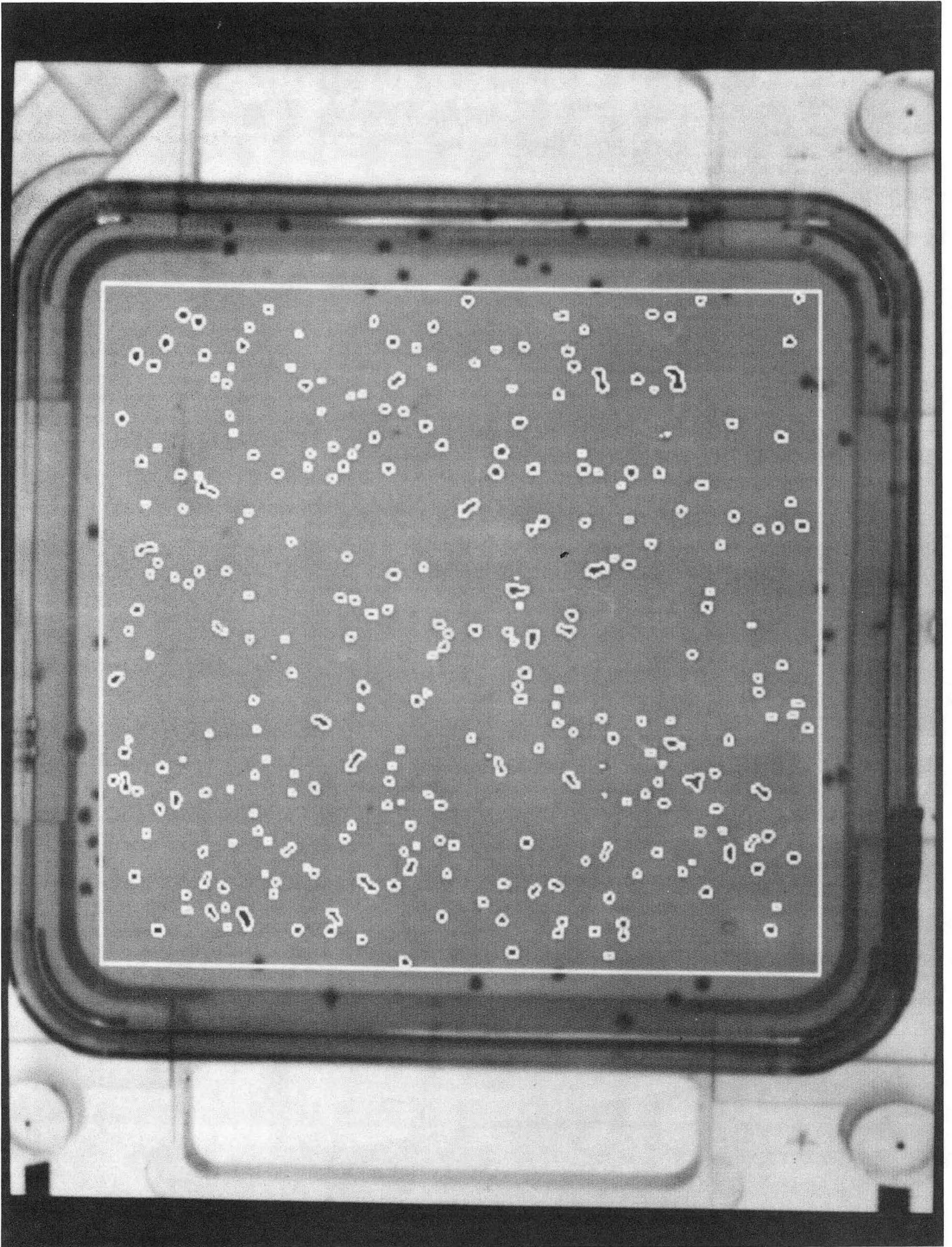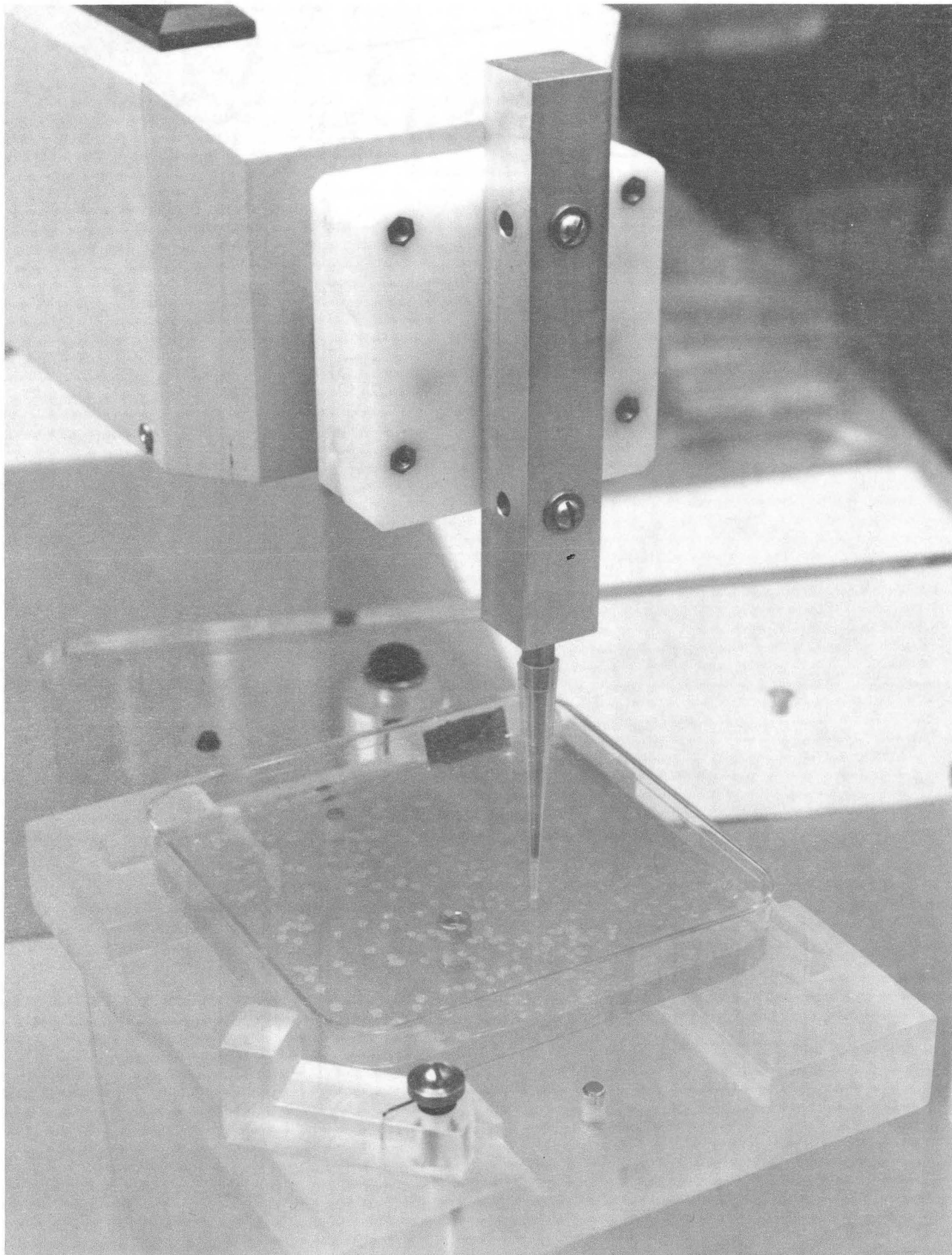
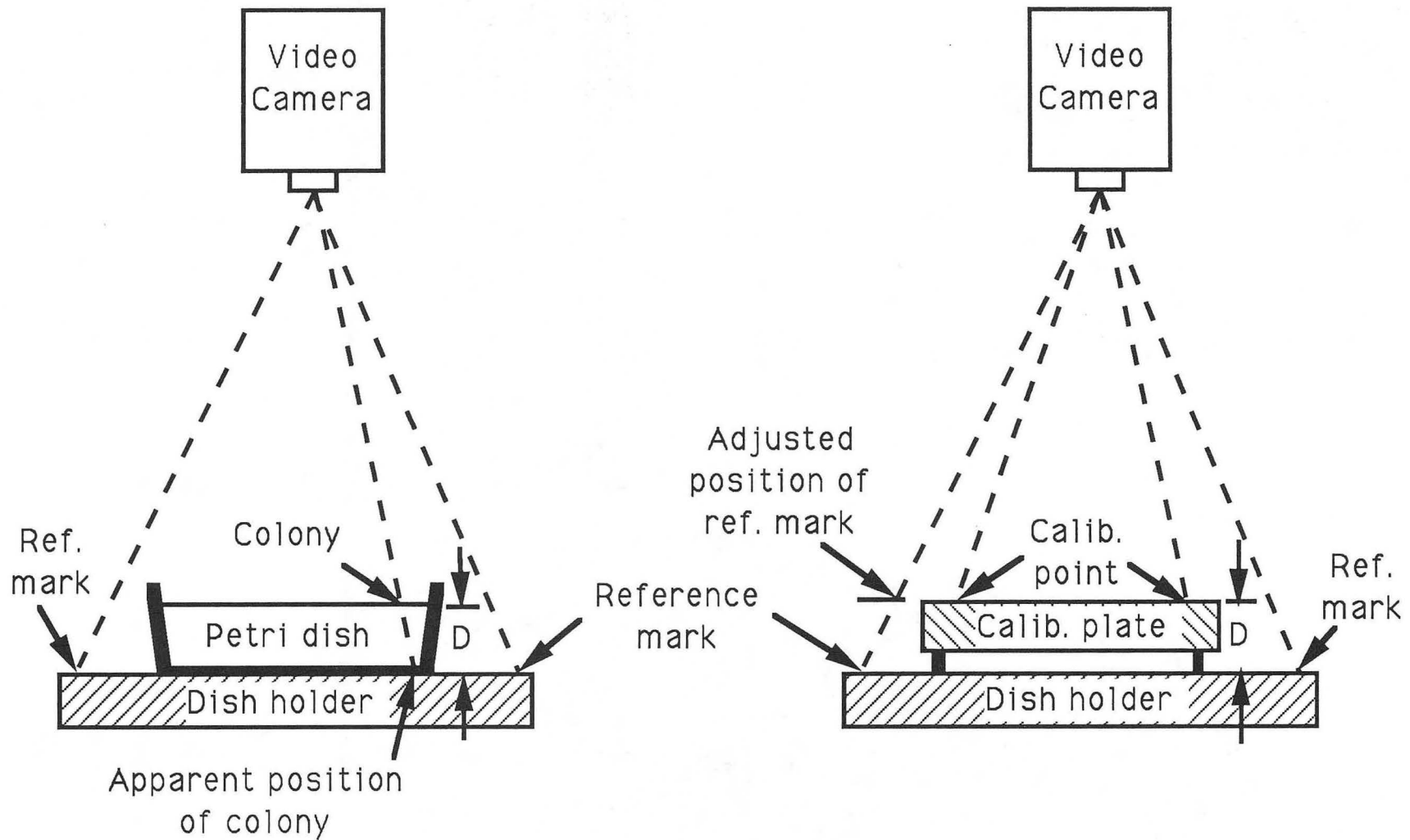FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

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