

sorting human chromosomes with flow cytometers and had been developing one capable of doing the job at a rate compatible with the scope of the project. We need something on the order of a million sorted chromosomes for each library. (Commercially available flow cytometers can sort that many in a few tens of hours—and that's operating time, so the job actually takes much longer—but the high-speed sorter reduces the sorting time to just a few hours. We made copies of the basic components of our sorter and sent them here. I understand the Los Alamos machine is now up and running.

As for Los Alamos, they brought to the project greater experience and expertise in recombinant DNA technology and in fact had made a few chromosome-specific libraries for the Chinese hamster before the project even started. The Los Alamos group had also explored the use of hybrid cells as sources of human chromosomes.

Our first joint meeting, at Los Alamos in December of '84, was proof to me of a successful collaboration. Almost everyone involved in the project at Livermore, from Ph.D.s to technicians, attended the meeting and took the opportunity to discuss common problems and exchange tricks of the trade with their counterparts at Los Alamos. Our chief cell farmer, for instance, had never been to Los Alamos to talk to the chief cell farmer here. They both do the same thing with somewhat different techniques and have developed somewhat different ways of getting around the many nitty-gritty problems that crop up in the business of cell culture. Together they did two experiments during the meeting. One day they grew cells and isolated the chromosomes using the technique that's favored at Livermore, and on the next day they went through the same steps using the technique favored at Los Alamos. Each learned something from the other and seemed very happy about the whole experience.

**SCIENCE:** *How much interest has been shown in the libraries?*

## Making the Libraries

# Supplying the Chromosomes

**A**s raw material for each chromosome-specific library, we must collect between half a million and a few million of a particular chromosome by sorting through tens to hundreds of millions with a flow cytometer. Our original hope was to obtain such large numbers of chromosomes for sorting by culturing, or multiplying *in vitro*, human cells, namely, fibroblasts from human foreskin tissue. But, as discussed below, these cells proved to be an acceptable source for only the smaller chromosomes, and another source had to be found for the larger.

We culture fibroblasts in three stages, beginning by placing small pieces of the tissue sample, together with nutrients essential for growth, in a small plastic container. (Despite the translation of *in vitro*, plastic has largely supplanted glass as a surface for cell culture.) The fibroblasts migrate from the tissue, adhere to the bottom of the container, and multiply by mitosis. (This process is similar to that by which fibroblasts *in vivo* repair a break in the skin.) Mitosis stops

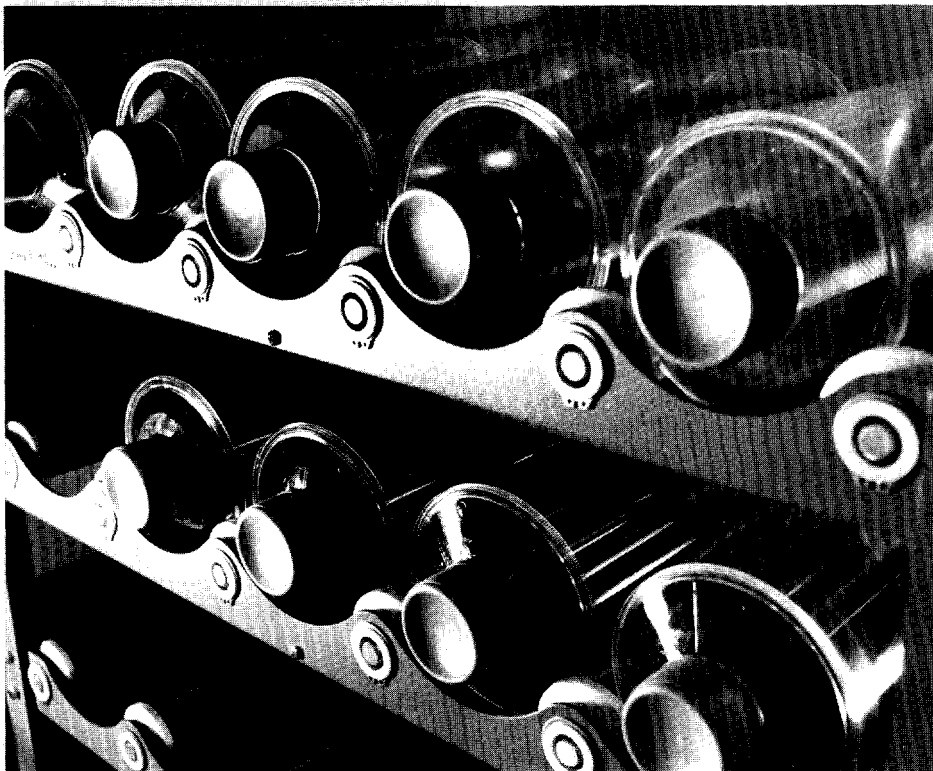
when the bottom of the container is covered with a monolayer of cells (Fig. 1). The monolayer is then partially digested with the enzyme trypsin to yield a suspension of single cells. This suspension is divided among several containers, fresh growth medium is added, and the culturing is repeated, again until a monolayer has formed.

Our aim is to maximize the number of fibroblasts at metaphase (on the verge of dividing), since only those cells contain the metaphase chromosomes that can be sorted. Obviously, the more rapidly a population of cells is multiplying, the larger is the fraction of that population at metaphase. But the fibroblasts begin to multiply less rapidly after about fifteen to twenty mitoses and cease multiplying altogether after about fifty. Therefore, after the second culture some of the fibroblasts are suspended in a medium containing glycerol (or some other ice-crystal inhibitor) and frozen in liquid nitrogen. These cells, which can be thawed and recultured at any time up to several years later, serve as a reservoir of fibroblasts at the height

I. SUPPLYING THE CHROMOSOMES



*Fig. 1. Cells in culture multiply by mitosis and spread over the surface of the culture dish until a confluent monolayer of cells is formed. Shown here is an optical micrograph of a confluent monolayer of fibroblasts cultured from a piece of human skin tissue (dark area).*



*Fig. 2. Cells can multiply manifold in large, slowly rotating cylinders ("roller bottles") since a large surface area is kept in contact with the culture medium.*

## I. SUPPLYING THE CHROMOSOMES

of their multiplication rate.

The final culture of the remaining fibroblasts takes place in large "roller bottles" (Fig. 2). When a monolayer has almost formed, the drug Colcemid is added to arrest the cell cycle at metaphase. After exposure to this drug for an appropriate time, the fraction of cells at metaphase increases from the usual maximum of 0.5 percent to between 30 and 50 percent. Since the metaphase fibroblasts "round up" from the culture surface because of their more spherical shape, they are easily separated by gentle shaking of the roller bottles.

The next step is to isolate the chromosomes from the cells. First, the cells are swollen in an "isolation buffer" with an osmotic pressure lower than that of physiological saline. The buffer is then forced through a fine-gauge hypodermic needle to break the cell membranes (the nuclear membranes are already dissipated in metaphase cells) and release the chromosomes. The buffer solution also contains materials, such as proteins that coat chromosomes or dyes that intercalate with DNA, to stabilize the morphology of the chromosomes. Although this isolation step sounds simple in principle, difficulties that plague even the best methods are responsible for our yield of only 2 to 6 sorted chromosomes from every 100 cells.

The final step in preparing the chromosomes for sorting is addition to the buffer of two fluorescent dyes. One of these binds preferentially to DNA sequences rich in adenine-thymine base pairs and the other to sequences rich in guanine-cytosine base pairs. The fluorescence intensities excited in the dyes serve as the basis for flow-cytometric analysis and sorting of the chromosomes.

Analysis of chromosome preparations from human fibroblasts indicated that all the chromosomes except numbers 9 through 12 were sufficiently well resolved for sorting (see Fig. 2 in "Sorting the Chromosomes"). In practice, however, sorting for the larger chromosomes was very time-consuming, apparently because the shearing action that breaks the cell membranes also breaks up

many of the larger chromosomes and greatly reduces their numbers. More important, the purities of these sorts were reduced to less than the acceptable level of 90 percent by many doublets of smaller chromosomes. Evidently the shearing action is not sufficient to break up these doublets, which form during isolation and cannot be distinguished by the flow cytometer from the larger chromosomes. Furthermore, reducing the shearing force to avoid breaking the larger chromosomes only increased the number of doublets.

As a result, we have had to turn to human-hamster hybrid cells as a source of the larger chromosomes. Such cells result from fusion of human and hamster cells and initially contain a complete set of both human and hamster chromosomes. However, after several weeks to several months in culture, the hybrid cells spontaneously and randomly lose some of their complement of human chromosomes. These losses make it possible to clone hybrid cells carrying selected human chromosomes. For our purpose an ideal hybrid cell is one with no more than three human chromosomes, each separable in a flow cytometer from each other and from the hamster chromosomes.

Since hybrid cells with a small number of human chromosomes are useful in other areas of research, various cell lines have been established and frozen at a number of laboratories. We are indeed grateful to those who have shared their cell lines with us for the Gene Library Project.

Culture of the hybrid cells is similar to, and no more difficult than, that of human fibroblasts. However, as sources of chromosomes for the libraries, hybrid cells are far from perfect. Their habit of unexpectedly losing human chromosomes, for instance, requires frequent monitoring for the continued presence of the desired chromosomes. Even more troubling is the fairly common occurrence of DNA exchange between the human and the hamster chromosomes. Such exchanges are difficult to detect but, undetected, contaminate the libraries with hamster genes. ■

**DEAVEN:** Well, we recently filled requests for over two hundred and fifty Phase I libraries from users in the United States and seven other countries. We expect many more requests as word of their availability gets around.

**MOYZIS:** I would be very surprised if [ever] research group in the world working on human genes wouldn't want at least one of the libraries, especially after the Phase II libraries are available.

**SCIENCE:** *Do you exercise any control over the uses to which the libraries are put?*

**DEAVEN:** No. The request form for a library includes a question about the nature of the proposed research, but we ask that primarily because we want to keep a record of the various applications. (Users funded by government agencies have already agreed to the guidelines established by the National Institutes of Health for recombinant DNA research, and industry voluntarily abides by those guidelines.)

Incidentally the plan for the future is that the NIH will establish a repository for the libraries we produce and will handle the distribution. They will also collect information determined by the users about the libraries, such as purity and completeness data and characteristics of probes isolated from the libraries. This information will serve as feedback to us for improving future libraries and will of course be valuable to other users. NIH also plans to establish a repository for probes pulled from our libraries and others.

**MOYZIS:** One thing we do request of users is that they don't pass the libraries on to other investigators. We want the libraries to originate directly from us, or from the repository when that comes about. One reason for this is so that we can keep in touch with all the users. A more important reason for not wanting the libraries passed around is to preserve their characteristics. Each amplification of a library unavoidably introduces changes. For example, since phages carrying human DNA fragment A do not multiply at exactly the same rate as those carrying fragment B, after several amplifications the rela-